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14. ABSTRACT
Metastatic spread of prostate cancer is the second leading cause of deaths of men in the United States. Although there are many ways to treat non-metastatic form of prostate cancer , only androgen-deprivation therapy is available for the extensive stage. Again, the cancer will often progress to an androgen refractory (independent), metastatic stage. Recent reports have suggested that the expression of VEGF-C is directly correlated with lymph node dissemination in prostate cancer. This finding leads us to think that understanding the role of angiogenic molecules like VEGF-C, -D in prostate cancer metastasis. Interestingly our results suggest a function of VEGF-C, which is directly related to its role in increasing the metastatic propensity of prostate cancer rather than inducing lymphangiogenesis.We have also delineated both positive (FOXO-1, ROS and RalA) and negative (NKX3.1) regulatory pathways that can be accounted for VEGF-C synthesis in prostate cancer cells.

15. SUBJECT TERMS
Vascular endothelial growth factor-C, lymphangiogenesis, metastasis

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Introduction: Metastatic spread of prostate cancer is the second leading cause of deaths of men in the United States. Every year almost 40.000 American men die from prostate cancer of which more than 70% die due to complications of late stage tumors that metastasize to distant location. Although there are many ways to treat non-metastatic form of prostate cancer like anti-androgen therapy, radical prostatectomy, radiotherapy and cytotherapy, only androgen-deprivation therapy is available for the extensive stage. Again, the cancer will often progress to an androgen refractory (independent), metastatic stage (1, 6, 17). However, the detailed molecular mechanism underlying the metastatic spread of this disease is poorly understood. Thus it has been difficult to develop effective treatments in this stage of prostate cancer. Recent reports have suggested that the expression of VEGF-C is directly correlated with lymph node dissemination in prostate cancer (7, 16). This finding leads us to think that understanding the role of angiogenic molecules like VEGF-C, -D in molecular detail in prostate cancer will provide us the information regarding their relationship with metastasis. Thus, in this present study, the main focus will be to unravel the detail molecular mechanisms of these molecules that lead to the metastatic spread of prostate cancer.

Body: Task1 described in the Statement of Work of our proposed application was as follows:

a. Develop a prostate cancer orthrotopic mouse model.

We successfully accomplished the goal by generating the orthotopic human prostate cancer mouse models by implanting the LNCaP, LNCaP C4-2 and PC3 cells. We have also made orthotopic mouse tumor model with LNCaP C4-2 stably expressing Luciferase gene. This model is helping us to determine the tumor growth in a non-invasive way. Details of these models development were mentioned in our previous annual reports. We are also collaborating with James Glockner and Erik L. Ritman of Mayo Clinic, Rochester to study the growth and metastasis of the tumor by MRI and MicroCT (as shown in Figure 1).

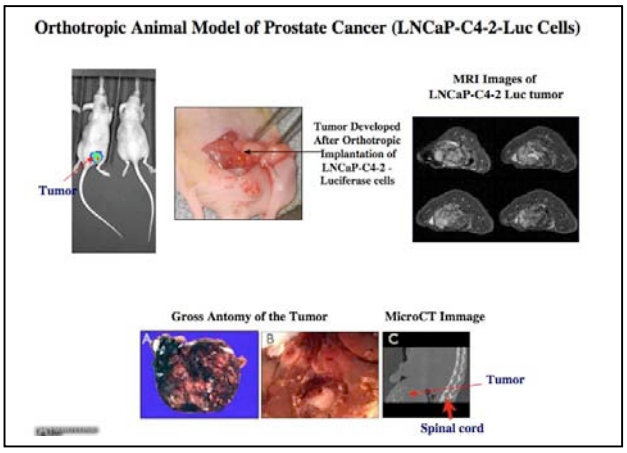


Figure 1: Orthotopic implantation of LNCaP C4-2-Luciferase cells in mice prostate.

b. ***Detection of lymphangiogenesis in prostate cancer and its relation to lymph metastasis.***

The extent to which lymphangiogenesis is required for prostate cancer lymph node metastasis remains unclear in light of several contradictory reports published recently. In our previous report, we provided detail discussion about those reports. In summary, we now understand that further studies are required to precisely determine the importance of lymphoangiogenesis in prostate cancer metastasis. In our previous annual report and also in our publication (8), we mentioned an increase in VEGF-C level during androgen ablated condition in prostate cancer cells indicating specific roles of VEGF-C during androgen refractory stage of the disease. In this light, the significance of lymphangiogenesis for promoting lymph node metastasis may not be crucial in the androgen refractory stage of prostate cancer, because systemic metastasis mainly occurs at this stage of the disease. Several reports and our recent work now point to other VEGF-C functions that are independent of lymphangiogenesis but nevertheless important for tumor

cell survival and metastasis. We believe that these functions of VEGF-C play significant roles in inducing tumor cell metastasis at the androgen refractory stage of prostate cancer.

In our previous report, we presented results that favor the autocrine function of VEGF-C in prostate cancer. They are as follows: We have detected neuropilin-2 and VEGFR2, two of the known receptors of VEGF-C, in different prostate cancer cell lines, suggesting a possible autocrine function for VEGF-C. With VEGF-C stimulation, we have observed an increase in the expression of the androgen receptor co-activator, Bag-1L in LNCaP cells (8). Bag-1L has been reported to enhance the trans-activation function of the androgen receptor with the help of Hsp70. Therefore; a VEGF-C-induced increase in Bag-1L indicates possible androgen receptor trans-activation even in the presence of low androgen concentrations, leading to the generation of a tumor with a more aggressive phenotype. Interestingly, we have observed an increase in the level of GTP-bound Rac (functionally active Rac) compared to total Rac protein in both androgen dependent (LNCaP) and androgen refractory (LNCaP C4-2) prostate cancer cells stimulated with VEGF-C. Rac activation is associated with phyllopodia formation in the protruding edge of motile cell. We have observed a decrease in the level of alpha-catenin in prostate cancer cells after VEGF-C stimulation. A decrease in alpha-catenin levels hinders E-cadherin and actin filament interaction and therefore disrupts cell-to-cell contact, which is required during cell migration.

Presently we have made the following observations that will help us to explain the molecular mechanisms of VEGF-C functions on prostate cancer cells.

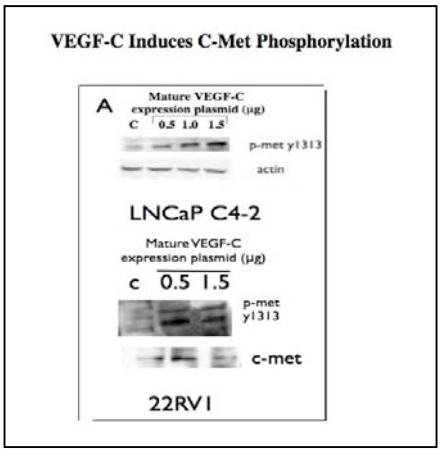


Figure 2: LNCaP and 22RV1 were transfected with VEGF-C expressing plasmid. Protein was harvested 48 hour after transfection followed by westernblot with p-Met 1313 antibody.

1. *Upon VEGF-C stimulation the tyrosine residue 1313 is phosphorylated in different prostate cancer cell lines.* We have observed phosphorylation of c-Met (a tyrosine kinase receptor known for its metastasis) in prostate cancer cells upon VEGF-C stimulation. To determine exactly the phosphorylation site of c-Met after VEGF-C treatment we checked different sites of c-met phosphorylation. Interestingly, the docking site of c-met (y1349 and y1356), which is mainly responsible for the effects of HGF, the only described ligand for c-Met does not show any change in phoshorylation status. Instead, the less-characterized tyrosine residue 1313 is responsible for the increase in c-met phosphorylation in different prostate cancer cell lines (LNCaP C4-2, 22RV1) (Figure 2). Y1313 has been shown to be a classical binding site for PI3-kinase. As PI3K is an activator of the Akt/PKB pathways in normal and cancer cells, we evaluated the status of Akt1 protein levels and Akt1 activation in prostate cancer cells after VEGF-C treatment or overexpression in our later experiments.

c-Met and Integrin β4 expression are associated with the metastatic potential of the prostate cancer cells.

We postulated that VEGF-C might phosphorylate c-met to enhance the metastatic properties of prostate carcinoma cells. To confirm this hypothesis we correlated c-Met and its important co-receptor Integrin β4 expression with the metastatic potential of syngenic LNCaP cell lines. In concordance with our hypothesis we were able to demonstrate that with increasing metastatic potential the levels of c-met

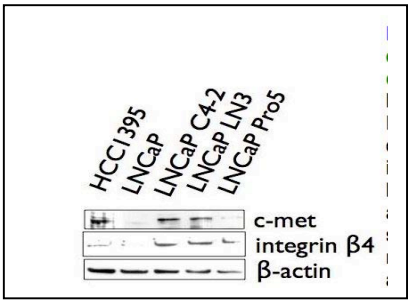


Figure 3: c-Met and integrin b4 expression in syngenic LNCaP cells demonstrated by immunoblot with the whole cell lysates.

Unpublished data

and its coreceptor Integrin β4 had increased (Figure 3). So, c-Met is highly expressed in metastatic cells and its activation by VEGF-C might further enhance metastasis.

VEGF-C regulates Akt1 in a dose and time dependent manner. With increasing doses of VEGF-C over time we observe an early upregulation and a late downregulation of Akt1 (Figure 4). The activation pattern of Akt1 (phosphorylation of Ser 473 as shown or Thr 308, data not shown) follows this pattern closely.

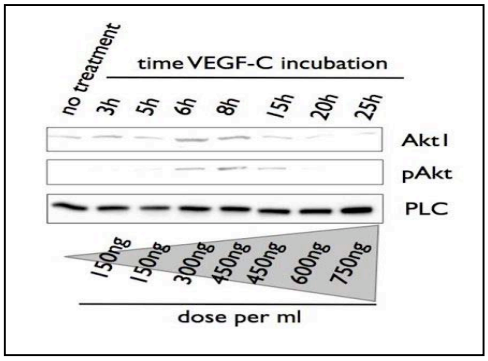
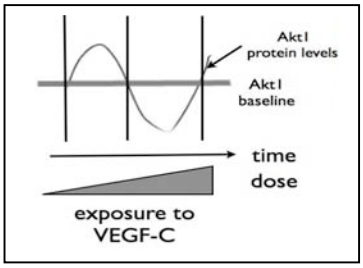


Figure 4: An early upregulation and a prominent late decrease in Akt1 protein levels at different time points and different dosages of VEGF-C in LNCaP C4-2 prostate cancer cells. Immunoblot of whole cell lysates after incubation with recombinant human VEGF-C. P-Akt1, phosphorylation at ser473 follows the pattern of Akt1 protein. PLCg serves as a loading control.

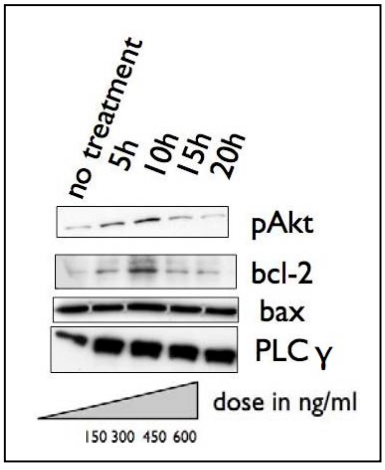
Interestingly, when treating the LNCaP C4-2 cells with a constant dose of VEGF-C at different time points, we could detect only an upregulation of Akt1 (data not shown).

When overexpressing mature VEGF-C in low doses, we could observe a pattern of Akt1 regulation, which resembles the result of constant doses over time (data not shown). In contrast, transfection with higher doses (1 μg) of the VEGF-C expression plasmid show an early upregulation and a late downregulation of Akt1 (data shown).



In summary, our data suggest that a wave like pattern of Akt1 regulation is induced by increasing doses of VEGF-C with time.

Akt kinases are critical and central mediators of signal transduction pathways downstream of tyrosine kinase receptors. Akt is well established as a promoter of cell survival and growth as well as angiogenesis in tumor cells (20). Furthermore, Akt has an important role in transmitting non-genomic signals involving the androgen receptor. It has been shown that Akt is amplified in hormone resistant prostate cancer (21). Recently, cytoplasmic overexpression of Akt1 was correlated with higher risk of PSA recurrence and shorter PSA recurrence intervals in an immunohistochemical study. Akt1 expression has even demonstrated to be an independent prognostic marker (22). Overall, an increasing number of patients show overexpression or overactivity of Akt during metastatic or androgen independent disease development. Interestingly, we found an upregulation of VEGF-C in prostate cancer with increased androgen independence of prostate cancer cells (8, 23). Neuropilin-2, one of the receptors for VEGF-C, is upregulated with increased metastatic potential, too. Accordingly, our results on VEGF-C dependent Akt1 regulation suggest, that VEGF-C might play an important role in the progression and metastasis of prostate cancer.



Significance of Akt1 upregulation:

VEGF-C mediated Akt1 regulation promotes survival by upregulation of Bcl-2. Akt inhibits proapoptotic factors such as BAD, BAX, and BID. The phosphorylation of BAD blocks degradation of bcl-2. Accordingly, our experiments show a clear upregulation of bcl-2 after VEGF-C incubation for about 10 hour (Figure 5). Bax expression does not show any alteration with time.

Figure 5: Bcl2 upregulation after VEGF-C incubation in LNCaP C4-2 prostate cancer cells. Immunoblot of the whole cell lysates after incubation with the recombinant VEGF-C

Unpublished data

Interestingly, upregulation of bcl2 is a frequent and important step in the progression to advanced and hormone independent disease. Bcl2 is expressed more strongly as malignant change progresses along the continuum from preinvasive to metastatic cancer (24). As a result, bcl2 emerges more and more as a important target for therapy using antisense bcl2 oligomers like oblimersen (25). VEGF-C induces bcl2 expression and thus protects tumor cells from apoptosis.

These results indicate that VEGF-C may increase the overall survival and the metastatic propensity of prostate tumor cells. A similar autocrine loop has recently been described by Su *et. al.,* (14) who showed that VEGF-C promotes the invasion and metastasis of lung and breast tumors.

Taken together, our results and other reports suggest that VEGF-C may stimulate an increase in the metastatic propensity of prostate cancer cells, which would also explain the observation that the peritumoral lymphatic infiltration of prostate cancer cells correlates with lymph node metastasis (19).
On the other hand, lymph node metastasis usually occurs in patients before the androgen refractory stage of the disease. Therefore, the increase in the VEGF-C level during androgen withdrawal therapy might increase the survival of prostate cancer cells by increasing androgen receptor trans-activation (increasing Bag1L) during their transition to the androgen refractory stage and also help to enhance their systemic metastasis (increasing migratory property).

Significance of Akt1 downregulation:

VEGF-C enhances migration by inhibiting NFAT degradation. The effects on apoptosis are most prominent in the time of Akt1 upregulation. Next, we evaluated the biologic significance of the Akt1 downregulation. Interestingly, an unexpected role of decreased activated Akt1 levels has been found in tumor metastasis (26). In breast cancer, Akt1 has been reported to suppress metastasis by increasing the levels of a transcription factor called NFAT (Nuclear Factor of Activated T-Cell). NFAT has shown to transduce signals from α6β4 integrin, which is also associated with c-Met (27). Accordingly, NFAT is important for invasion of breast carcinoma cells. Recently, NFAT has also been proved to be important for the metastatic progression of androgen independent prostate cancer, too (28). In breast cancer, Akt1 blocks cancer cell motility by facilitating the degradation of NFAT. Another study in breast cancer has reported that crossing MMTV/ activated Akt transgenic mice with an activated ErbB2 strain results in accelerated mammary tumorigenesis (29). However, these mice harbored fewer metastatic lesions than the activated ErbB2 strain alone. This result therefore suggests that although Akt1 promotes the progression of primary tumorigenesis of breast tumors, at a certain point may negatively regulate breast tumor metastasis. In respect to these results which show the importance of a downregulated active Akt1 in tumor invasion and metastasis by inhibiting NFAT degradation, we tested the protein levels of NFAT during the time points of Akt downregulation. In fact, we detected NFAT after substantial downregulation of activated Akt (Figure 6), which suggests an induction of invasion and metastasis in the phases of Akt downregulation by VEGF-C.

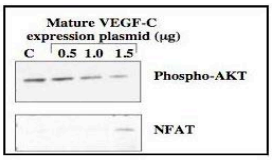


Figure 6: NFAT is upregulated after Akt downregulation. Immunoblot of the whole *cell lysates 96h after transfection with mature VEGF-C.*

Accordingly, we have observed an increase in the cell migration of the androgen refractory LNCaP C4-2 cells in a transwell cell migration assay with wild type recombinant VEGF-C stimulation (data not shown). *In summary, the wave like regulation pattern of Akt1 by VEGF-C make it possible for the cells to survive by blocking apoptosis mechanisms (upregulation of Akt1) and to invade and metastasize by upregulating promigratory proteins like NFAT (downregulation of Akt1).*

At present, we are working on to fulfill the following goals.

1. Preparation of stable LNCaP and LNCaP C4-2 cell lines that expresses full length and mature form of VEGF-C.
2. *In vivo* experiments for examining the effect of VEGF-C overexpressing prostate cancer cells for survival and metastasis.

Task 2. Elucidation of the molecular pathway that regulates the expression of VEGF-C in prostate cancer.

Transcriptional regulation of VEGF-C in prostate cancer

Despite this evidence for the role of VEGF-C in advanced stage prostate cancer, the molecular mechanism involved in VEGF-C expression is poorly understood. We have observed an increase in VEGF-C synthesis during androgen-ablated condition. We have delineated two possible molecular mechanisms that can explain the increase in VEGF-C expression in prostate cancer cells in the absence of androgen. Details of these mechanisms were described in our previous reports and our publications in the journal *Oncogene*. A summary of those signaling pathways has been described in figure 7.

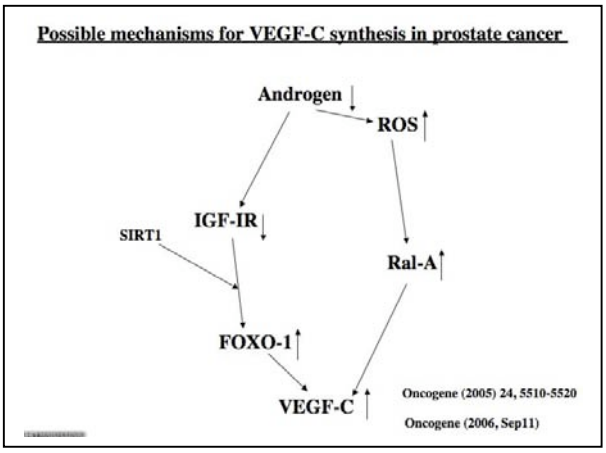


Figure 7: Signaling pathways involved in the upregulation of VEGF-C synthesis during androgen ablation in prostate cancer cells.

In our original proposal, we mentioned potential binding sites for prostate-specific homeodomain protein NKX3.1 in the VEGF-C promoter region (Figure 8). We postulated that by binding to the VEGF-C promoter region, NKX3.1acts as a transcriptional repressor of VEGF-C. NKX3.1 is required for normal prostate development and more importantly any NKX3.1 loss-of-function contributes to prostate carcinoma. Therefore, we hypothesize that loss of NKX3.1 during the initiation of prostate carcinoma also facilitates the synthesis of high level of VEGF-C in prostate cancer cells.

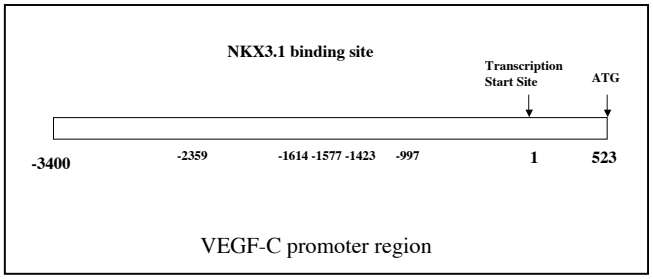


Figure 8: Schematic diagram of probable NKX3.1binding sites in the VEGF-C promoter region.

In this report, we are providing evidences that support our hypothesis of the inhibitory role of NKX3.1 on VEGF-C synthesis.

We performed chromatin Immunoprecipitation (CHIP) assay to understand the binding site of NKX3.1 in VEGF-C promoter (Figure 9). Our CHIP result (Figure) suggested the region at -997 of VEGF-C promoter actually binds

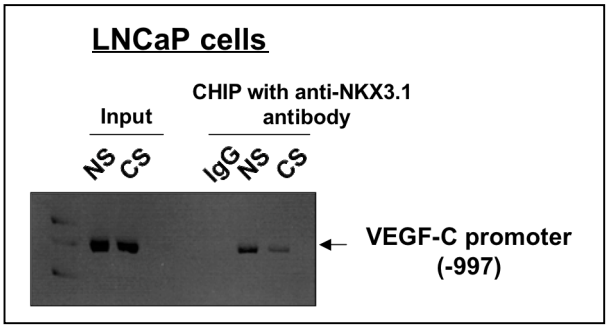


Figure 9: CHIP assay in LNCaP cells cultured in normal serum (NS) or charcoal-stripped serum (CS) to show NKX3.1 binding to the specific (-997) region of VEGF-C promoter.

Unpublished data

NKX3.1. We also observed less binding of NKX3.1 in VEGF-C promoter in LNCaP cells, which was cultured in the absence of androgen. Previous report suggested that androgen upregulated NKX3.1 level in LNCaP cells.

Interestingly, when we ectopically over expressed NKX3.1 in LNCaP cells, a dose dependent decrease in VEGF-C mRNA level was observed (Figure 10). This decrease in VEGF-C mRNA level by overexpressing NKX3.1 can be blocked by knocking down NKX3.1 level by siRNA (Figure 11). Similar decrease in VEGF-C mRNA level was observed in PC3 cells by overexpressing NKX3.1 (data not shown). All these results clearly proved the ability of NKX3.1 to decrease VEGF-C mRNA level.

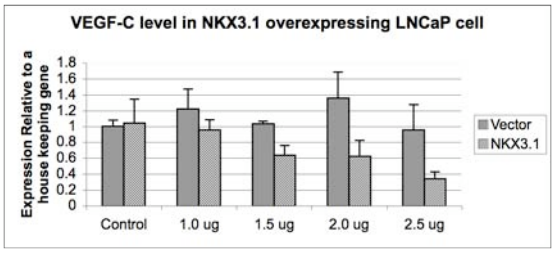


Figure 10: Real-time PCR to determine the VEGF-C mRNA level in LNCaP cells after overexpressing increasing doses of NKX3.1.

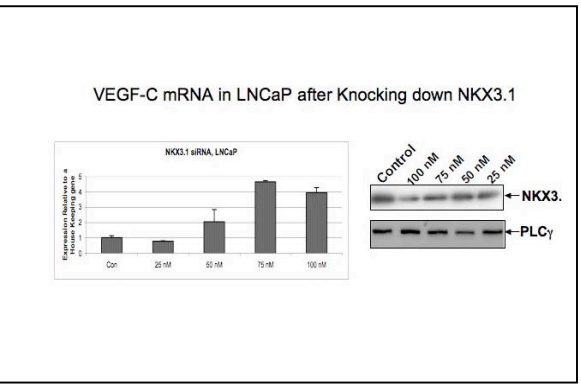


Figure 11: Real-time PCR to determine the VEGF-C mRNA level in NKX3.1 overexpressing LNCaP cells that also express increasing doses of NKX3.1siRNA.

Histone deacetylase 1 (HDAC1) was shown to interact with NKX3.1. We were therefore interested to determine whether HDAC plays any role in inhibiting NKX3.1 mediated inhibition of VEGF-C transcription. So far, we used a generic histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA), specific for class I and II HDAC in increasing doses in LNCaP cells after overexpressing NKX3.1. We observed that SAHA could withdraw the inhibitory effect of NKX3.1 on VEGF-C transcription similar to siRNA of NKX3.1 (Figure 12). This data suggests the involvement of HDAC class I or II in inhibiting VEGF-C transcription in prostate cancer cell along with NKX3.1.

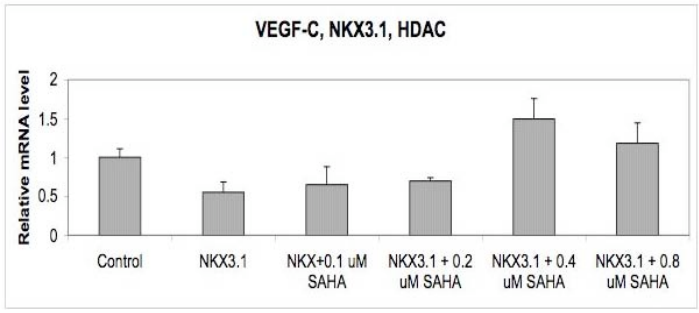


Figure 12: Real-time PCR to determine the VEGF-C mRNA level in NKX3.1 overexpressing LNCaP cells that were treated with increasing doses of SAHA.

Currently, we are performing experiments to determine the specific HDAC that might be involved with NKX3.1 for inhibiting VEGF-C transcription in prostate cancer cell.

Key Research Accomplishments:

1. We have successfully developed orthotopic mouse models of human prostate cancer that will enable us to study the role of VEGF-C in prostate cancer metastasis. We have also developed luciferase gene expressing prostate cancer cells that are helping us to image the tumor in the mouse in a non-invasive way.
2. We have observed functions of VEGF-C in increasing the metastatic propensity of prostate cancer cells. These functions of VEGF-C are distinct from its known function of inducing lymphangiogenesis.

3. We have delineated molecular pathways, which involve the involvement of reactive oxygen species and small GTPase RalA and FOXO-1 for the transcriptional upregulation of VEGF-C in prostate cancer in the androgen-ablated condition. We have also determined the role of NKX3.1 for transcriptional repression of VEGF-C in prostate cancer cells.

Reportable Outcome:

Published paper:

Rinaldo F, Li J, Wang E, Muders M, **Datta K** (2007) RalA regulates vascular endothelial growth factor-C (VEGF-C) synthesis in prostate cancer cell during androgen ablation. *Oncogene* 26(12),1731-8

Li J, Wang E, Rinaldo F, **Datta K**. (2005) Up-regulation of VEGF-C by androgen depletion: the involvement of IGF-IR-FOXO pathway. *Oncogene* 24(35), 5510-20.

Abstract presented:
Rinaldo F, Muders M, Li J, **Datta K.** (2007) Synthesis and Function of Vascular Endothelial Growth Factor-C during Androgen Ablation. *Innovative Minds in Prostate Cancer Today (IMPACT)*, Atlanta, Georgia.
Li J, Wang E, Rinaldo F, **Datta K**. (2006) Regulation and Function of Vascular Endothelial Growth Factor-C in Prostate Cancer. *Prostate Spore Meeting*, Houston, Texas.
Li J, Wang E, Rinaldo F, **Datta K**. (2006) Regulation and Function of Vascular Endothelial Growth Factor-C in Prostate Cancer. *12th Spore Workshop*, Bultimore.

Plasmids generated: Generated LNCaP-C4-2-luciferase stable cell line, VEGF-C promoter luciferase expression construct in pGL3basic. siRNA of VEGF-C in pRetroSuper and pRetroSupirior.
Developed the orthotopic mouse model of human prostate cancer in immunocompromised mice.
Developed stable luciferase and dsRed fluorence protein expressing prostate cancer cells.
Developed stable VEGF-C overexpressing LNCaP C4-2 cells

List of personnel: Kaustubh Datta, Jinping Li, Enfeng Wang, Francesca Rinaldo, Haoji Huang, Janice Nagy

Conclusion:

1. We were able to develop the orthotopic mouse model of prostate cancer. Further progress has been made by creating stable cell lines that express luciferase gene and dsRed fluorence protein and by studying the growth of prostate cancer in a non-invasive way. Currently, microCT and MRI are being performed on this model to understand the growth and metastasis of the cancer in a non-invasive way.
2. We have observed autocrine function of VEGF-C in prostate cancer cells that might be important for its transition to androgen refractory, metastatic stage.
3. We have also delineated positive (FOXO1, ROS, RalA) and negative (NKX3.1) regulatory pathways for VEGF-C synthesis in prostate cancer cells.

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Upregulation of VEGF-C by androgen depletion: the involvement of IGF-IR-FOXO pathway

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Androgen ablation therapy is eventually followed by a more metastatic and androgen-refractory stage of prostate cancer. The detailed molecular mechanism of this gradual transition is not clearly understood. Recent reports correlate the high abundance of vascular endothelial growth factor-C (VEGF-C) to the lymph node metastasis seen in human prostate cancer (Tsurusaki *et al*., 1999). In this study, we report that androgen ablation in LNCaP cells augment the transcriptional upregulation of VEGF-C and the downregulation of the IGF-IR pathway, due to androgen withdrawal, is a potential mechanism for this observed VEGF-C transcription. Forkhead transcription factor FOXO-1, activated by SIRT-1, was identified as the downstream molecule within this pathway. Furthermore, the VEGF-C-induced increase of Bag-IL expression in LNCaP cells suggests that VEGF-C plays a role in the androgen-independent reactivation of the androgen receptor, resulting in androgen-refractory prostate cancer growth.
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Keywords: VEGF-C; prostate cancer; metastasis; androgen; IGF-IR

Introduction

Prostate cancer is the second leading cause of cancer-related mortality for men in the United States (Greenlee *et al*., 2000). The established protocol for treating the advanced stage of this disease is surgical castration and/or chemotherapy to eliminate circulating androgens (Epstein *et al*., 1996). Activation of the androgen receptor by androgens is required for cell proliferation in the prostate. Therefore, androgen ablation therapy is initially effective in inhibiting cancer cell growth in most patients. However after a period of time, the tumor recurs in an androgen-refractory manner with a more aggressive and metastatic phenotype, ultimately leading to patient death (Kyprianou *et al*., 1990; Denis and Murphy, 1993; Oh and Kantoff, 1998; Isaacs, 1999). As the detailed molecular mechanism underlying the metastatic spread of prostate cancer is poorly understood, it has therefore been difficult to develop effective treatments at this stage of the disease. Initially, the concept of clonal selection was thought to explain the progression of the disease but subsequent molecular investigations of the androgen receptor (AR) provided a more mechanistic approach to understanding the metastatic progression of prostate cancer during androgen deprivation. Possible mechanisms for AR re-activation include AR gene mutation, gene amplification, the involvement of coregulators, and crosstalk between different signal transduction pathways (Sadar *et al*., 1999).

Apart from the possibilities discussed above, the involvement of other pathological mechanisms cannot be dismissed. In this regard, angiogenesis represents an effective target mechanism (Stewart *et al*., 2001; Nicholson and Theodorescu, 2004; Gustavsson *et al*., 2005). Angiogenesis is a process by which new vasculature is developed from pre-existing vessels and it is particularly important for the growth and metastasis of most human tumors (Folkman, 1971; Holash *et al*., 1999; Carmeliet, 2000). There have been previous reports that androgens may potentially modulate angiogenesis in animal and human prostate tumors by increasing vascular endothelial growth factor (VEGF) gene transcription (Stewart *et al*., 2001). As a result, an initial and rapid vascular regression in the tumor occurs after hormone withdrawal. Interestingly, progression of prostate cancer to an androgen-independent stage is also associated with increased angiogenesis. The lack of angiogenic regulation by androgens may be an additional factor accompanying the transition to this stage of androgen resistance (Jain *et al*., 1998). Therefore, it is important to identify any regulatory dysfunction of angiogenic growth factors during androgen-refractory stage of prostate cancer.

VEGF-A is the founding member of closely related cytokines that exert critical functions in vasculogenesis, pathologic and physiologic angiogenesis and lymphangiogenesis (Dvorak *et al*., 1995; Dvorak, 2000; Ferrara, 2001). Other members of the VEGF family include VEGF-B, VEGF-C, VEGF-D, VEGF-E (orf virus VEGF) and placental growth factor (P1GF) (Chilov

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et al., 1997; Enholm *et al*., 1997; Achen *et al*., 1998, 2002; Olofsson *et al*., 1999; Dvorak, 2000; Carmeliet *et al*., 2001). All of these ligands mainly bind to and activate three receptor tyrosine kinases (TRK) to initiate their downstream signaling. These TRKs include: VEGFR-l/Flt-1, VEGFR-2/Flk-l/KDR and VEGFR-3/Flt4. VEGF-A binds to VEGFR-1 and VEGFR-2 whereas VEGF-C and -D bind to VEGFR-2 and VEGFR-3. VEGF-B binds only VEGFR-1 and VEGF-E binds only VEGFR-2 (de Vries *et al*., 1992; Dvorak *et al*., 1995; Hatva *et al*., 1995; Kaipainen *et al*., 1995; Joukov *et al*., 1996; Dvorak, 2000; Kubo *et al*., 2000; Veikkola *et al*., 2000). There is also a fourth nontyrosine kinase receptor called neuropilin-1 that mainly binds VEGF-A (Soker *et al*, 1998).

Among the angiogenic cytokines, VEGF-C draws special attention because of its potential involvement in the lymph node metastasis of several cancers including – colorectal cancer, human pancreatic endocrine tumors, esophageal carcinomas, head and neck squamous cell carcinoma, uterine cervical cancer, primary non-small-cell lung cancer, gastric carcinoma, and laryngeal squamous carcinoma (Yonemura *et al*., 1999; Hashimoto *et al*., 2001; Kimura *et al*., 2003; Neuchrist *et al*., 2003; Jia *et al*., 2004; Sipos *et al*., 2004; Wang *et al*., 2004). Recently, Tsurusaki *et al*. reported a significant correlation between the expression of VEGF-C and lymph node dissemination in human prostate carcinoma (Tsurusaki *et al*., 1999). They found an increased number of vessels positive for VEGFR-3, the VEGF-C receptor, in surrounding stromal tissue of VEGF-C positive prostatic carcinoma cells (Tsurusaki *et al*., 1999; Zeng *et al*., 2004). VEGF-C has a high degree of sequence homology with VEGF-A. It also has eight-conserved cysteine residues involved in intra- and intermolecular disulfide bonding. The cysteine-rich COOH-terminal increases the length of VEGF-C by half as compared to other VEGF family members. VEGF-C, like VEGF-A, has alternative spliced forms. VEGF-C mRNA is first translated into a precursor that, after secretion, undergoes cell associated proteolytic processing. The mature form of VEGF-C is attained after the proteolytic removal of its NH_2- and COOH-terminal extensions. Mature form has increased binding affinity towards VEGFR-3 by approximately 400-fold. It can also bind to VEGFR-2 while the unprocessed form of VEGF-C can bind only VEGFR-3 (Lee *et al*., 1996; Pepper, 2001). The mature form can induce endothelial cell proliferation and migration as well as vascular permeability (Joukov *et al*., 1997). Despite these reports, the individual role of each receptor on VEGF-C stimulation is not yet clear. In contrast to VEGF-A, VEGF-C is not regulated by hypoxia (Enholm *et al*., 1997). The expression profile of VEGF-C and its binding to VEGFR-3 suggests its involvement in the development of the lymphatic system or lymphangiogenesis in the tumor microenvironment.

Cytokines such as VEGF-C are multifunctional in nature. Therefore, increased expression of VEGF-C in the tumor microenvironment will trigger an enhancement of many cellular functions and deregulate the balance between different physiological processes. One possible function of VEGF-C in the tumor microenvironment mentioned before is that of lymphangiogenesis, providing a potential route for tumor cell dissemination to the lymph node. Because, this may not be the only function of VEGF-C-promoting metastasis, it will be interesting to study the potential functions of VEGF-C, apart from lymphangiogenesis, that may be responsible for the progression of prostate cancer to the androgen-refractory condition. It has been reported that VEGF-C can stimulate proliferation and migration of Kaposi's sarcoma cells (Marchio *et al*., 1999) and also proliferation and survival for leukemia (Dias *et al*., 2002). Again, lack of lymphangiogenesis observed in uveal melanoma despite its high expression of VEGF-C, suggests a different function in cancer progression (Clarijs *et al*., 2001).

In the androgen-dependent prostate cancer cell line LNCaP, we have observed VEGF-C expression. Furthermore, androgen withdrawal upregulates VEGF-C expression in LNCaP cells. Our data suggest that the androgen receptor (AR)-IGF-IR axis is important for the observed VEGF-C transcriptional upregulation in LNCaP. Again, an increase in Bag-1L protein level by VEGF-C was observed in LNCaP cells that may provide an explanation for activation of androgen receptor by VEGF-C in low androgen concentration.

Results

VEGF-C is highly expressed in metastatic prostate cancer cell line

Previous reports suggest a positive correlation between VEGF-C expression and the metastatic potential of human prostate cancer cells. In order to test whether a greater metastatic potential correlates with higher expression levels of VEGF-C, we used real-time PCR to quantify VEGF-C mRNA levels in three prostate cancer cell lines: PC-3, DU145, and LNCaP (Figure 1a). The VEGF-C mRNA level in each cell line was normalized to the housekeeping gene 36B4. Interestingly, the VEGF-C mRNA level in PC-3, the cell line with the highest metastatic potential, was 250-fold higher than that of LNCaP, the cell line with the lowest metastatic potential. DU145, intermediate in terms of metastatic potential, expressed VEGF-C mRNA at levels 150-fold greater than that of LNCaP but lower when compared to that of PC-3. Although VEGF-A mRNA levels were only two fold higher in the PC-3 cell line and four fold higher in the DU145 with respect to LNCaP, the levels were clearly not as high as VEGF-C (Figure 1b). Protein levels of VEGF-C in PC-3 and LNCaP cell lines were also in line with the real-time PCR data when tested by ELISA (Figure 1c). Taken together, these results pointed to the potential importance of VEGF-C in prostate cancer metastasis.

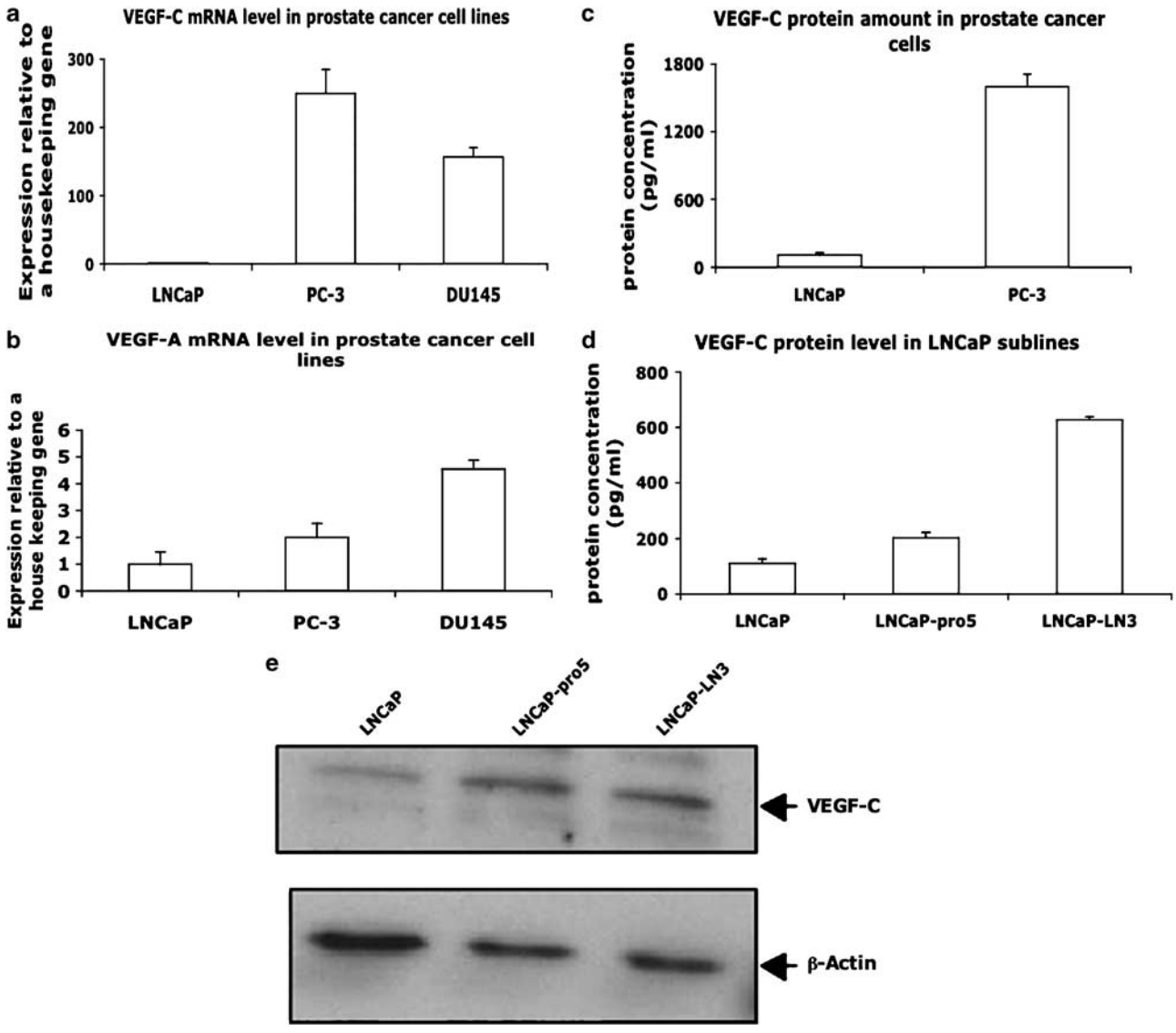

Figure 1 (**a** and **b**) mRNA level of VEGF-C and VEGF-A in LNCaP, DU145 and PC-3 cell lines. Real-time PCR was performed using specific primers for (**a**) VEGF-C, and (**b**) VEGF-A with total RNA isolated from three prostate cancer cell lines LNCaP, DU145 and PC-3. The data represented here are the average of three independent results. (**c**) Protein amount of VEGF-C in prostate cancer cell lines by ELISA. The amount of VEGF-C in the conditioned media of LNCaP and PC-3 cells was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using specific antibody targeting VEGF-C. The data presented are the mean of two independent experiments. (**d** and **e**) VEGF-C protein level in LNCap sublines. (**d**) The amount of VEGF-C in the conditioned media of LNCaP, LNCaP-Pro5 and LNCaP-LN3 cells was measured by ELISA using specific antibody against VEGF-C. The data presented are the mean of two independent experiments. (**e**) Whole cell extract was collected from LNCaP, LNCaP-Pro5 and LNCaP-LN3 cells. Western blot against VEGF-C (upper panel) antibody and β-actin (lower panel) was performed

The more metastatic subline LNCaP expresses higher levels of VEGF-C

VEGF-C protein levels were also tested in LNCaP-Pro5 and LNCaP-LN3, two different sublines of LNCaP with a greater metastatic potential than the parental cell line (Pettaway *et al*., 1996). We performed ELISA to quantitate the VEGF-C protein amount in the conditioned media of different LNCaP sublines (Figure 1d). The most metastatic subline LNCaP-LN3 expressed highest amount of VEGF-C. LNCaP-pro5 was intermediate in terms of metastatic potential among the sublines and expressed more VEGF-C protein than the parental cell line but less compared to LNCaP-LN3. Western blot results (Figure 1e) also show higher VEGF-C protein levels in both LNCaP-Pro5 and LNCaP-LN3 compared to the parental LNCaP line. These results again suggested the potential involvement of VEGF-C in metastasis.

Androgen negatively regulates VEGF-C levels in LNCaP cells

It is known that androgen deprivation is associated with the gradual transition of prostate cancer from the androgen-dependent stage to the androgen-refractory stage. Furthermore, the tumor recurs with a more

metastatic phenotype. This prompted us to investigate whether androgen can regulate VEGF-C expression in LNCaP cells. LNCaP cells were grown in media with charcoal–dextran-treated serum (reduced androgen-containing serum) for 48, 72, 96, and 120 h. Using real-time PCR, we observed a time-dependent upregulation of VEGF-C mRNA across the time points tested (Figure 2a), suggesting a negative regulation of VEGF-C expression by androgen. The value of VEGF-C mRNA level at each time point was normalized with the value of the house keeping gene 36B4. This result was confirmed when the upregulation of VEGF-C mRNA level was significantly inhibited after the addition of synthetic androgen (R1881) to the charcoal-treated media at the 48-h time point (Figure 2b). Additionally, the negative regulation of VEGF-C by androgen appears specific because a different profile was observed for VEGF-A mRNA. VEGF-A mRNA levels decreased in the absence of androgen but increased significantly when R1881 was added to the media (Figure 2c).

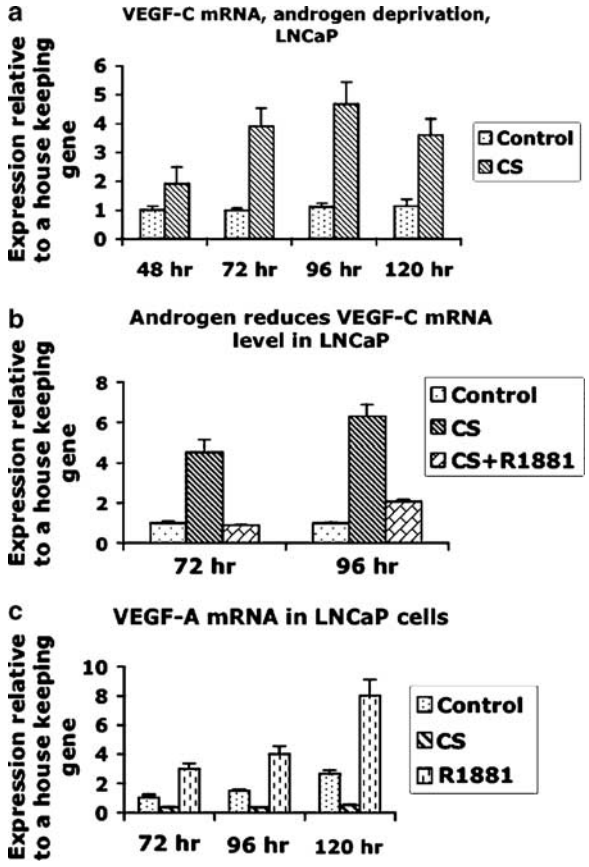

Figure 2 Androgen depletion increases VEGF-C mRNA level but decreases VEGF-A mRNA in LNCaP cells. (**a**) LNCaP cells were cultured in charcoal–dextran-treated serum-containing media (designated as CS in the figure) for 48, 72, 96 and 120 h. Total RNAs were obtained in each time point followed by RT–PCR using primers specific for VEGF-C and 36B4 (internal control). For the control experiment, LNCaP cells were cultured in the normal serum-containing media for the same time points described above and VEGF-C mRNA level was determined by RT–PCR. The data showed here are the average of three independent results. (**b** and **c**) LNCaP cells were first cultured in charcoal–dextran-treated serum-containing media (designated as CS in the figure) for 48 h. R1881 (1 μM) was then added to the media and the total RNA was isolated at the different time points with respect to the cells cultured in charcoal-stripped media. RT–PCR was performed with the total mRNA by using primers specific for VEGF-C (**b**) and specific for VEGF-A (**c**). 36B4 mRNA levels were monitored in every experiment for internal control. The data showed here are the average of three independent results

Functional inhibition of insulin-like growth factor-I (IGF-IR) signaling upregulates VEGF-C mRNA level

Recently, Arnold *et al*. (2005) described a functional correlation between androgen and IGF-IR expression in prostate cancer cells. They showed that androgen stimulation of LNCaP cells cultured in media containing charcoal–dextran-treated serum led to an increase in IGF-IR protein expression. We also observed the similar regulation of androgen for IGF-IR expression in LNCaP as shown in (Figure 3a). This result suggests the possible regulation of IGF-IR synthesis by androgens. In another report, Plymate *et al*. (2004) described that the progression from an organ-confined tumor to metastatic prostate cancer was characterized by a deregulation of the androgen receptor and a decrease in IGF-IR protein expression. These two findings suggest that decreasing IGF-IR levels plays a role on the progression of prostate cancer to its androgen-refractory, metastatic stage. Since androgen withdrawal also increases VEGF-C mRNA levels, we investigated whether IGF-IR also influenced regulation of VEGF-C transcription. Our real-time PCR data (Figure 3b) suggest a 8–10-fold increase in VEGF-C mRNA when blocking antibody specific for the IGF-IR receptor (Keller *et al*., 1993; De Meyts *et al*., 1995; Datta *et al*., 2000; Schlessinger, 2000) inhibited IGF-IR signaling. We also observed the increase in VEGF-C mRNA level in both the LNCaP-Pro5 and LNCaP-LN3 cells by inhibiting IGF-IR signaling (Figure 3c and d). The reason for not detecting the 8–10-fold increase in VEGF-C mRNA like parental LNCaP cell line was probably due to the fact that these two cell lines are more metastatic than LNCaP cells and already express higher level of VEGF-C (Figure 1d). Again, we did not observe any significant increase in VEGF-A mRNA in LNCaP cells when we inhibited the IGF-IR pathway suggesting a VEGF-C-specific phenomenon (Figure 3e). These results, therefore, correlate a possible link between androgen withdrawals, a decrease in IGF-IR protein expression, and VEGF-C upregulation in prostate cancer cells.

FOXO-1 is the potential transcription factor for VEGF-C and is activated by SIRT-1

Forkhead transcription factors become functionally active due to the downregulation of IGF-IR signaling. PKB or AKT activation by the IGF-IR-phosphoinositide-3 kinase (PI3K) pathway leads to the phosphorylation of forkhead transcription factors, which retains them in the cytoplasm (Borkhardt *et al*., 1997; Kops and

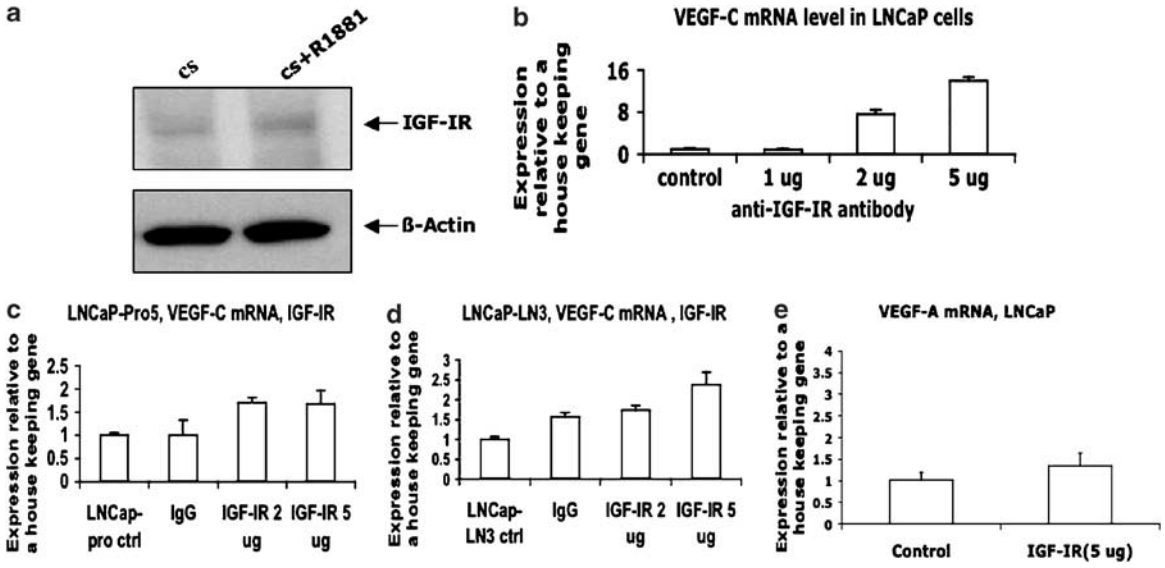

Figure 3 (**a**) Androgen increases the IGF-IR protein level in LNCaP. LNCaP cells were grown in the charcoal–dextran-treated serum-containing media (designated as CS in the figure) for 48 h, then R1881 (1 μM) was added for 24 h. Whole cell extracts were subjected to Western blot using anti-IGF-IR antibody. Protein level of IGF-IR was increased in presence of R1881 (upper panel), β-actin was used as internal control (lower panel). (**b**–**d**) Blocking IGF-IR signaling increases VEGF-C mRNA level in LNCaP, LNCaP-Pro5, and LNCaP-LN3 cells. LNCaP, LNCaP-Pro5, and LNCaP-LN3 cells were grown in serum-starved condition media for 24 h, treated with anti-IGF-IR blocking antibody at different concentrations (1, 2 and 5 μg/ml respectively) for overnight. Normal mouse IgG (5 μg/ml) was used as control. Real-time PCR was performed using primers of VEGF-C and 36B4 (internal control) with total RNA isolated from cells. In the figure, IgG stands for normal mouse IgG antibody and IGF-IR stands for anti-IGF-IR blocking antibody. The data here represent the mean of three different experiments. (**e**) VEGF-A mRNA level was unchanged in the presence of IGF-IR antibody. LNCaP cells were grown in serum-starved media for 24 h before treated with anti-IGF-IR-blocking antibody at different doses (1, 2 and 5 μg/ml respectively) for overnight. Normal mouse IgG (5 μg/ml) was used as control. Real-time PCR was performed using primers specific for VEGF-A and 36B4 (internal control) with total RNA isolated from LNCaP cells. The data here represent the mean of three different experiments

Burgering, 1999; Lin *et al*., 1997; Morris *et al*., 1996; Ogg *et al*., 1997). Therefore, inhibition of the IGF-IR signaling pathway should lead to the less phosphorylation of forkhead transcription factors, allowing them to enter the nucleus and promote the transcription of their target gene. Using the Genomatix software available on the internet (http://www.genomatix.de), we searched the known promoter region of VEGF-C for potential binding sites of transcriptional regulators and found several for the forkhead transcription factor, FOXO-1. To study whether FOXO-1 could indeed upregulate VEGF-C transcription, we transiently overexpressed FOXO-1 in LNCaP cells and used real-time PCR to monitor VEGF-C mRNA levels (Figure 4a). With the transient overexpression FOXO-1, we found a significant increase in VEGF-C mRNA levels. FOXO-1 protein overexpression was detected by Western blot with the anti-FLAG antibody (Figure 4b).

Various biological functions controlled by FOXO-1 include cell cycle arrest, detoxification of reactive oxygen species, repair of damaged DNA, and apoptosis (Kops and Burgering, 1999). Recent reports suggest that the presence of SIRT1 (mammalian homolog of yeast silencing information regulator 2 (Sir2) gene) enhances the expression of the FOXO target genes involved in stress resistance but diminishes the expression of proapoptotic genes like Fas ligand and BIM. Sir2 has been shown to play a role in increasing the lifespan of yeast and *Caenorhabditis elegans* and is conserved throughout evolution. It is a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase. Forkhead transcription factors have been shown to be a target of SIRT1. Deacetylation of FOXO transcription factors by SIRT1 is specifically required for the transcription of stress resistance genes, such as genes that control the repair of damaged DNA (GADD45) (Araki *et al*., 2004; Brunet *et al*., 2004; Cohen *et al*., 2004; Daitoku *et al*., 2004). Because the VEGF family is known to be involved in lymphangiogenesis, angiogenesis, and survival, we studied the involvement of SIRT1 in the FOXO-1-dependent transcription of VEGF-C. We found that BML-210, a specific SIRT1 inhibitor, abrogated FOXO-1 dependent VEGF-C transcription (Figure 4c). This result was confirmed when we used the dominant-negative form of SIRT1 to inhibit its activity (Figure 4d). SIRT-1DN protein overexpression was detected by Western blot with the anti-FLAG antibody (Figure 4e). Since androgen withdrawal leads to inhibition of PI3K-AKT pathway (Baron *et al*., 2004; Castoria *et al*., 2004; Kang *et al*., 2004) and therefore potentially activates FOXO-1, we tested whether dominant-negative form of SIRT-1 could abrogate the increase in VEGF-C expression in LNCaP due to androgen withdrawal. Our result (Figure 4f) clearly indicated the involvement of SIRT-1 in androgen-regulated VEGF-C transcription. These results are significant as they provide a mechanistic explanation for how FOXO-1, activated by SIRT1, can act to

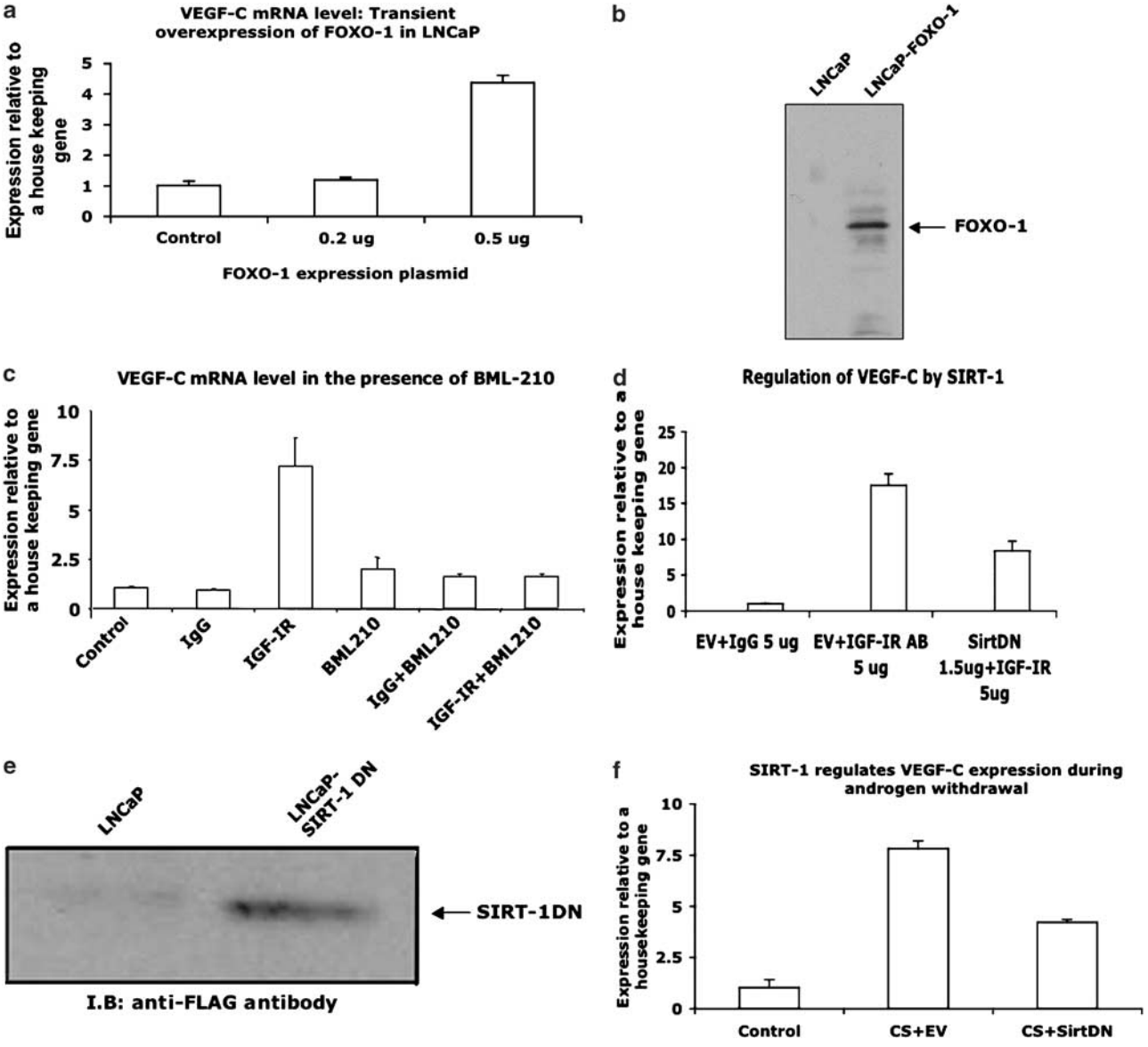

Figure 4 FOXO-1 with SIRT1 upregulates VEGF-C transcription. (**a**) LNCaP cells transiently transfected with pcDNA3–Flag–FOXO-l plasmid at 0.2 and 0.5 μg for 48 h, total RNA were subjected to real-time PCR using specific primers of VEGF-C and 36B4 (internal control), an increased mRNA level of VEGF-C was observed in LNCaP cells where FOXO-1 was overexpressed. The data here represent mean of three separate assays. (**b**) Western blot was performed using anti-flag antibody with the whole cell lysate extracted from the LNCaP cells transiently transfected with 0.5 μg of Flag-FOXO-1 plasmid. (**c**) FOXO-1-dependent VEGF-C transcription is abrogated by BML-210 (SIRT1 inhibitor). LNCaP cells were grown in 0.1% FBS for overnight. Anti-IGF-IR blocking antibody (5 μg/ml) either alone or along with BML-210 (10 μM) was then added for 8 h. Normal mouse IgG (5 μg/ml) was also used separately either alone or along with BML-210 (10 μM) for control experiment. Total RNA was isolated for each set and real time PCR was performed for VEGF-C and 36B4 (internal control). In the figure, IgG stands for normal mouse IgG antibody and IGF-IR stands for anti-IGF-IR blocking antibody. The data here represent mean of three separate assays. (**d**) Overexpression of dominant-negative form of SIRTl decrease the VEGF-C mRNA level. LNCaP cells transiently transfected with plasmid expressing dominant-negative form of SIRT1 (1.5 μg, pECE-SIRT-lDN)) in the presence or absence of anti-IGF-IR antibody (5 μg/ml). Total RNA was extracted from LNCaP cells and mRNA level of VEGF-C was determined by RT–PCR using primers specific for VEGF-C and 36B4 (internal control). In the figure, EV represents empty vector, pECE. IgG stands for normal mouse IgG antibody and IGF-IR stands for anti-IGF-IR blocking antibody. The data here represent mean of three separate assays. (**e**) Western blot was performed using anti-flag antibody with the whole cell lysate extracted from the LNCaP cells transiently transfected with 1.5 μg of Flag-SIRT-IDN plasmid. (**f**) Overexpression of dominant-negative form of SIRT1 decrease the VEGF-C mRNA level induced by androgen depletion. LNCaP cells transiently transfected with either plasmid expressing dominant-negative form of SIRT1 (1.5 μg) or empty vector (EV) and cultured in the presence of charcoal–dextran-treated serum-containing media for 72 h. For control experiment, LNCaP cells were cultured in the normal serum-containing media. Total RNA was extracted from LNCaP cells and mRNA level of VEGF-C was determined by RT–PCR using primers specific for VEGF-C and 36B4 (internal control). The data here represent mean of three separate assays

transcribe VEGF-C under androgen withdrawal condition and it also indicates that VEGF-C is another target gene for SIRT1.

VEGF-C can increase the expression of the transcription coactivator BAG-IL in LNCaP

VEGF family members are multifunctional growth factor proteins. The established function of VEGF-C is to potentiate the growth and sprouting of lymphatic endothelial cells or lymphangiogenesis by binding its receptor VEGFR-3. It is therefore important to determine the contribution of lymphangiogenesis to the survival and metastasis of prostate cancer cells during their transition to the androgen-refractory stage. In a recent report, Rafi *et al*. showed that expression of the antiapoptotic protein Bcl-2 was increased in the leukemia cells after treatment with VEGF-C (Dias *et al*., 2002) suggesting a function distinct from lymphangiogenesis. In the prostate cancer cell line LNCaP, the mRNA level of VEGFR3 was low and at least 10-fold lower than VEGFR2 as detected by real-time PCR (Figure 5a). The relative expression levels of these two receptors in the lymphatic endothelial cells (kind gift from Dr Mihaela Skobe) were also tested as a control experiment (Figure 5b). Figure 5a therefore indicated a potential VEGF-C–VEGFR-2-mediated autocrine pathways in LNCaP. A similar autocrine loop for VEGF-A–VEGFR2 in LNCaP cells were also reported by Steiner *et al*. (2004). Although VEGF-C is known to bind VEGFR-2, the significance of this binding is not known. Interestingly, we observed an increase in the expression of Bag-1 long isoform (Bag-IL) after treating serum-starved LNCaP cells with recombinant VEGF-C protein for 24 h (Figure 5c). Several reports suggested that the transcriptional coactivator, Bag-IL enhances the transactivation function of androgen receptor (Froesch *et al*., 1998; Shatkina *et al*., 2003). Therefore, an increase in Bag-IL protein level by VEGF-C may therefore provide an explanation of VEGF-C-mediated androgen receptor activation in the low concentration of androgen.

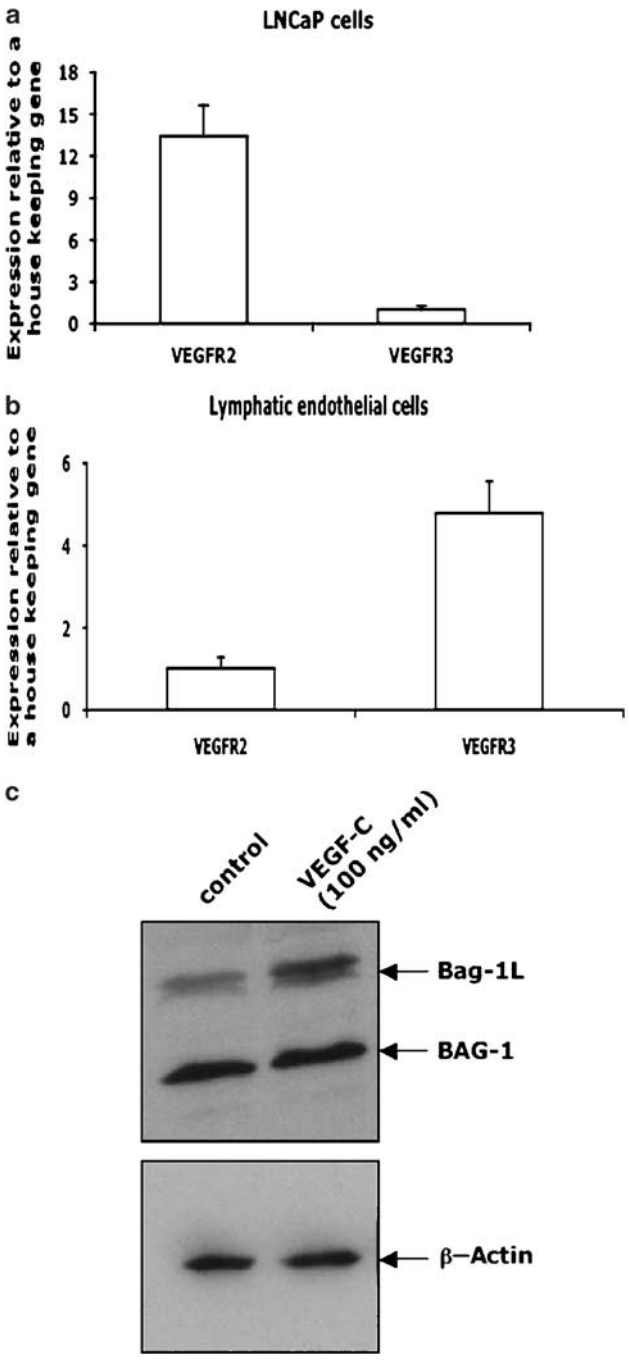

Figure 5 (**a** and **b**) VEGFR2 is predominant in LNCaP cells compare to VEGFR3. Real-time PCR was performed using specific primers for VEGFR2, and VEGFR3 with total RNA isolated from (**a**) LNCaP cells and (**b**) lymphatic endothelial cells (kindly provided by Dr Mihaela Skobe). The data represented here are the average of three independent results. (**c**) Recombinant VEGF-C protein increases the protein level of long isoform of Bag-1 (BaglL). Serum-starved LNCaP cells were incubated with recombinant VEGF-C protein (100 ng/ml) for 24 h. The proteins of the whole cell lysates were resolved by SDS–PAGE followed by Western blot using antibody specific for Bag-1. This antibody also recognizes Bag-IL (the long isoform) at 58 kDa. Membrane was then stripped for Western blot against β-actin (lower panel). The Western blot was repeated more than three times

Discussion

A detail molecular understanding of the gradual transition of prostate tumors from androgen dependence to androgen independence during androgen ablation therapy is necessary to design an effective therapy against this stage of the disease. Although some mechanisms have been postulated for androgen receptor activation, the involvement of angiogenic growth factors provides an alternative avenue of study. It is possible that angiogenic growth factors may act independently or in conjugation with the androgen receptor, to regulate the survival and metastasis of the androgen-refractory stage of prostate cancer. The possible crosstalk between the androgen receptor and angiogenic growth factors was first established by observing that androgens may modulate angiogenesis in animal and human prostate tumors, potentially by increasing vascular endothelial

growth factor-A (VEGF-A) gene transcription (Jain *et al*., 1998; Stewart *et al*., 2001). As a result, an initial and rapid vascular regression occurs after hormone withdrawal in prostate cancer. Surprisingly, however, progression of prostate cancer into an androgen-independent stage is also associated with increased angiogenesis. Thus far, the mechanism of this second wave of angiogenesis is unknown.

Interestingly, VEGF-C, another VEGF family member, is also expressed in human metastatic prostate cancer specimens. An increased number of vessels positive for VEGFR-3, the VEGF-C receptor, has been reported in the surrounding stromal tissue of VEGF-C positive prostatic carcinoma cells (Skobe *et al*., 2001; Zeng *et al*., 2004). Taken together, these reports suggest the importance of VEGF-C in prostate cancer metastasis. We have observed an increase in VEGF-C mRNA and protein in the more metastatic prostate cancer cell lines PC3 and DU145 as compared to LNCaP. Even the more metastatic sublines of LNCaP, LNCaP-LN3 and LNCaP-Pro5 expressed higher levels of VEGF-C compared to the parental line. Our focus in this study was to understand the mechanism of VEGF-C expression in prostate cancer, specifically in the context of androgen-deprivation conditions. We observed a time-dependent increase in VEGF-C mRNA levels when LNCaP cells were cultured in media containing charcoal–dextran-treated serum (reduced androgen containing serum). This increase in VEGF-C mRNA levels was inhibited after supplementing the media with the synthetic androgen R1881. This result therefore confirms the previous findings (Ruohola *et al*., 1999) that androgens have a negative regulatory effect on VEGF-C expression and suggests a potential functional significance for higher VEGF-C levels during the initial stage of androgen withdrawal. Interestingly, we observed a decrease in VEGF-A mRNA levels in LNCaP cells upon androgen depletion, confirming previous findings that androgen positively regulates VEGF-A mRNA transcription. These observations describe a potentially unique role for VEGF-C in androgen regulation.

Insight into a possible cellular mechanism for the elevation of VEGF-C levels under androgen-depleted conditions came with the observation that inhibition of the IGF-IR signaling pathway in LNCaP cells lead to the upregulation of VEGF-C mRNA. A recent report suggested that androgen stimulation of LNCaP cells cultured in media containing charcoal–dextran-treated serum leads to an increase in IGF-IR protein expression (Arnold *et al*., 2005). The activation of PI3K pathway by androgen was also reported previously (Baron *et al*., 2004; Castoria *et al*., 2004; Kang *et al*., 2004). These reports, together with our observation of higher VEGF-C mRNA levels after inhibiting IGF-IR signaling supports the conclusion that androgen deprivation leads to a decrease in IGF-IR expression that in turn increases the transcription of VEGF-C. This negative regulation of VEGF-C transcription by IGF-IR might be specific for prostate cancer. Recently, Tang *et al*. (2003) demonstrated a positive regulation of VEGF-C transcription by IGF-IR in Lewis lung carcinoma subline, M27. These differences in IGF-IR signaling for VEGF-C transcription therefore pointed out the heterogeneity of cellular signaling in different cancers and also in the different stages of a particular cancer. FOXO-1 is a member of the forkhead transcription factor family whose transcriptional activity is negatively regulated by IGF-IR (Morris *et al*., 1996; Borkhardt *et al*., 1997; Lin *et al*., 1997; Ogg *et al*., 1997; Kops and Burgering, 1999) and is therefore a potential downstream target of IGF-IR in VEGF-C transcription. Interestingly, we have found several potential binding sites for FOXO-1 in the VEGF-C promoter region using Genomatix software. To show that FOXO-1 is the probable transcription factor for VEGF-C, we have overexpressed FOXO-1 in LNCaP cells. A significant increase in VEGF-C mRNA was observed in LNCaP cells overexpressing FOXO-1. The target genes for FOXO-1 include both the proapoptotic genes as well as the genes involved in stress resistance. Recently, a protein called SIRT1 has been shown to regulate FOXO-1 activity by enhancing the transcription of stress resistance genes but diminished the expression of proapoptotic genes such as Fas ligand and BIM. SIRT1 specifically deacetylates FOXO-1 in order to regulate its activity (Araki *et al*., 2004; Brunet *et al*., 2004; Cohen *et al*., 2004; Daitoku *et al*., 2004). The finding that VEGF-C transcription driven by FOXO-1 requires SIRT1 suggests that VEGF-C is another survival related target gene of FOXO-1. In this study, we have therefore suggested a possible mechanism of VEGF-C transcription resulting from androgen ablation in the prostate cancer cell line, LNCaP. Androgen ablation decreases IGF-IR expression and therefore disrupts its function. The resulting effect is activation of FOXO-1, which with SIRT1, increases the transcriptional activity of VEGF-C.

VEGF-C can potentiate different functions in prostate cancer, many of which may be responsible for its growth and survival in the androgen-withdrawal stage. Particularly, increased synthesis of VEGF-C may be linked to its early and mid-phase transition to the highly metastatic, and androgen-refractory stage. The main function of VEGF-C is its involvement in lymphangiogenesis where the growth and sprouting of lymphatic endothelium occurs from a pre-existing lymphatic vessel (Skobe *et al*., 2001). Increased lymphatic vessel formation surrounding a tumor may provide potential routes for tumor cells to metastasize to distant organs. As androgen withdrawal triggers tumor growth arrest and tumor apoptosis in the primary site, an increase in the metastatic potential therefore helps the tumor cells to migrate to a different location and survive. Interestingly, there are reports that suggest a significant correlation between the expression of VEGF-C and lymph node dissemination in human prostate carcinoma (Tsurusaki *et al*., 1999). Moreover, the number of vessels positive for VEGFR-3, the receptor of VEGF-C, was increased in the surrounding stromal tissue of VEGF-C-positive prostatic carcinoma cells (Tsurusaki *et al*., 1999). Therefore, it will be interesting to study the involvement of lymphangiogenesis in prostate cancer metastasis during its transition to the androgen-refractory stage.

As VEGF family members are usually multifunctional in nature, we were also interested in identifying VEGF-C functions distinct from lymphangiogenesis that may be responsible for prostate cancer progression. We tested for the presence of VEGFR2 and VEGFR3, two receptors for VEGF-C in LNCaP cell lines with real-time PCR. We detected a very low level of VEGFR-3 in LNCaP cells, but VEGFR-2 was in the detectable level and $\sim$10-fold higher than VEGFR-3 under our experimental conditions (Figure 5a). This result suggests a possible VEGF-C autocrine loop in prostate cancer cells and possibly some VEGFR-2 specific functions in LNCaP. The concept of autocrine loop for VEGF family members in prostate cancer cells were further supported by the recent report of Steiner *et al*. (2004). The authors reported VEGF-A–VEGFR2-mediated autocrine loop in LNCaP cells after prolonged IL6 treatment. Along these lines, LNCaP cells also provide a unique opportunity to study the specific functions of VEGF-C and VEGFR-2 that are currently unclear.

The finding that BAG-IL (long isoform of Bag-1) was increased in serum-starved LNCaP after VEGF-C treatment is also significant. Bag-IL is a member of the Bcl-2 family and plays a role in cell survival during stress conditions. Bag-l(Bcl-2-associated athanogene 1) is a family of proteins that associates with the molecular chaperone Hsp70 and serves as a nucleotide exchange factor for this protein. There are four mammalian isoforms of Bag-1; Bag-lL, Bag-IM, Bag-lS, and p29, generated by alternative translation initiation sites on the same mRNA. The human Bag-IL protein migrates as a 57–58 kDa protein in SDS–PAGE experiments. The Bag-1 proteins have multiple biological functions ranging from inhibition of apoptosis to modulation of the action of steroid receptors. Bag-IL has been reported to enhance the transactivation function of the androgen receptor with the help of Hsp70. Furthermore, Bag-IL is expressed in prostate cancer specimens (Froesch *et al*., 1998; Sadar *et al*., 1999; Shatkina *et al*., 2003). Therefore, a VEGF-C-induced increase in Bag-IL may enhance transactivation of androgen receptor even in low concentrations of androgen.

In conclusion, we have observed an increase in the VEGF-C level in prostate cancer cells due to the withdrawal of androgens. We have delineated a possible mechanism for VEGF-C synthesis in LNCaP cells involving the androgen–IGF-IR–FOXO-1 pathway. Finally, we also determined a potential function mediated by VEGF-C that may be important for the growth and survival of prostate cancer from the early phase of its transition to the androgen-refractory stage.

Materials and methods

Cell culture and reagents

Human prostate cancer cell line, LNCaP (ATCC # CRL-1740), DU-145 (ATCC # HTB-81), and PC-3 (ATCC #CRL-1435), were maintained in RPMI medium and Ham's F-12 medium separately with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA). LNCap-LN3 and LNCap-pro5 were kind gifts from Dr Curtis A Pettaway of MD Anderson. FOXO-1 expression vector was obtained from Dr Haoji Huang of Mayo Clinic. Human SIRT1 dominant-negative (DN) plasmid (pECE-SIRTlDN) was also a kind gift from Dr Michael E Greenberg, Children's Hospital and Harvard Medical School, Boston. *NLP-005* Methyltrienolone (R1881) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BML-210 was purchased from BIOMOL International L.P. (Plymouth Meeting, PA, USA).

Transfection and whole cell extract preparation

LNCaP cells (5×10^5 cells) were seeded in 60 mm dish 1 day before transfection. Transfection was carried out with Effectene Transfection Kit (Qiagen, Valencia, CA, USA). Briefly, 0.5 μg of the FOXO-1 expression plasmid was resuspended in EC buffer (75 μl) and 4 μl of Enhancer was added and incubated at room temperature for 5 min. Effectene (10 μl) was then added and the whole mixture was incubated for another 10 min. RPMI with 10% FBS (350 μl) was added to the DNA mixtures. At 48 h after transfection, cells were washed three times with PBS, whole cell extracts was prepared in accordance to the following protocol.

Preparation of whole cell extracts

LNCaP, DU145, PC-3, LNCap-LN3 and LNCap-Pro5 cells were washed twice with 10 ml of cold PBS, lysed with ice-cold RIPA lysis buffer (50 mM Tris (pH 7.5), 1% Nonidet p-40 (NP-40), 150 mM NaCl, 1 mM Na_3VO_4, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 mg/ml), 0.5% aprotinin, 2 mM pepstatin A), incubated on ice for 30 min, and centrifuged at 14 000 r.p.m. at 4°C for 10 min.

Western blot analysis

The whole cell extracts were separated by SDS–PAGE; immunodetection antibodies against VEGF-C (R&D System, Inc., Minneapolis, MN, USA), Flag (Sigma), IGF-IRα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Bag-1 (Zymed Laboratories Inc., South San Francisco, CA, USA) were used, followed by the secondary antibody incubation, and detected by ESL enhancer reagent from Amersham Biosciences Corp (Piscataway, NJ, USA).

ELISA

Cell culture supernatant was collected after growing the prostate cancer cells at serum-starved media for 24 h. VEGF-C was measured in the cell culture supernatant using a commercial ELISA kit (Zymed laboratories Inc.) according to the manufacturer's protocol. Briefly, after incubation 100 μl of sample media or different dilutions of standard in the plate at 37°C for 1 h, the wells were washed three times with washing buffer, 100 μl of labeled antibody solution was added to each well for a 30 min incubation at 4°C. For color development, TMB buffer (100 μl) was added into the wells and was incubated for 30 min at room temperature in dark, the reaction was stopped by adding 100 μl of Stop solution. Color intensity was measured by a plate reader at 450 nm. Data represent the average of three different assays.

Treatment of cells with antibody, androgen and specific SIRT inhibitor

Cells were pretreated with IGF-IRα antibody (1H7) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (Keller *et al*., 1993; De Meyts *et al*., 1995; Datta *et al*., 2000; Schlessinger,

2000), R1881, BML-210 (specific SIRT inhibitor) and recombinant VEGF-C protein at 37°C for different time points. Cells were serum starved before IGF-IRa antibody, BML-210 and recombinant VEGF-C protein treatment. For R1881, cells were cultured in charcoal–dextran-treated serum for 48 h. After washing twice with ice-cold PBS, cells were lysed using lysis buffer from the RNeasy Mini kit (Qiagen). Total RNA was extracted according to the RNeasy mini kit protocol.

RNA preparation and real-time PCR

After washing twice with ice-cold PBS, different cell lines LNCaP and other cells were lysed using lysis buffer from the RNeasy Mini kit (Qiagen). Total RNA was extracted according to the RNeasy mini kit protocol. We used the Taqman real-time PCR method. The sequence for forward, reverse and Taqman middle primers for human VEGF-C, VEGF-A, VEGFR2, VEGFR3 and for human 36B4 (housekeeping gene) were taken from the PubMed gene bank and synthesized (Integrated DNA Technology). 36B4 is a ribosomal RNA and ubiquitously expressed in all the cells. Owing to its housekeeping function, it is used as a control experiment for real-time PCR, Northern blot etc. VEGF-C forward: 5′- AGT GTC AGG CAG CGA ACA AGA -3′. VEGF-C reverse: 5′-CCT CCT GAG CCA GGC ATC TG -3′. VEGF-C middle primer: 5′- TGC CCC ACC AAT TAC ATG TGG AAT AAT CA -3′. VEGF-A forward: 5′-TAC CTC CAC CAT GCC AAG TG-3′. VEGF-A reverse: 5′-GAT GAT TCT GCC CTC CTC CTT-3′. VEGF-A middle primer: 5′-TCC CAG GCT GCA CCC ATG GC -3′. VEGFR2 forward: 5′-CAC CAC TCA AAC GCT GAC ATG TA-3′. VEGFR2 reverse: 5′-CCA ACT GCC AAT ACC AGT GGA-3′. VEGFR2 middle primer: 5′-TGC CAT TCC TCC CCC GCA TC-3′. VEGFR3 forward: 5′-CAA GGC CAA CAA CGG CAT-3′. VEGFR3 reverse: 5′-TCG ACG CTG ATG AAG GGG ATT-3′. VEGFR3 middle primer: 5′-CAC AAT GAC CTC GGT GCT CTC CCG-3′. 36B4 forward: 5′-ATG CAG CAG ATC CGC ATG T-3′. 36B4 reverse: 5′-TCA TGG TGT TCT TGC CCA TCA-3′. 36B4 middle primer: 5′-CAC CAC AGC CTT CCC GCG AA-3′. Each real-time PCR reaction was conducted using 0.5 mg total RNA, 25 μl RT–PCR Master Mix (Applied Biosystems), 1.25 μl RNase inhibitor (Applied Biosystems), 50 nM forward primer, 50 nM reverse primer, and 100 nM middle primer. For reverse transcription, a 30-min period at 48°C was run before inactivating the reverse transcriptase at 95°C for 10 min. A total of 40 cycles at 95°C for 15 s and 60°C for 1 min was performed with an ABI Prism 7700 Sequence Detector (Applied Biosystems). All experiments were carried out three times and from each of the three, triplicate readings were taken and the average was calculated.

Relative RNA amount was calculated as follows: The cycle number (CT) at which PCR amplification took place in a particular threshold value in the exponential phase for each reaction was determined by the sequence detector. Real-time PCR for the housekeeping gene, 36B4 was performed for each test sample along with VEGF-C or VEGF-A. To normalize the value of VEGF-C or VEGF-A for each reaction condition, the value of 36B4 at that condition was deducted and the resulting value was designated as Δ. Therefore, $\Delta = \mathrm{CT}$ (VEGF-C or A sample)$-$CT(36B4 sample). To calculate the relative expression of the treated sample compare to the control sample, first $\Delta\Delta$ was determined by deducting the Δ value of the control sample from the treated sample. Therefore, $\Delta\Delta = \Delta$ (transfected or treated sample)$-\Delta$(empty vector or untreated sample). Finally, relative RNA amount in comparison to the control was calculated by using the formula, $2^{-\Delta\Delta}$. Average and standard deviations from three experiments were calculated.

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ORIGINAL ARTICLE

RalA regulates vascular endothelial growth factor-C (VEGF-C) synthesis in prostate cancer cells during androgen ablation

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Prostate cancer mortality is primarily due to failure to cure patients with metastatic disease. In its early stages, prostate cancer growth is enhanced by androgens. As such, the primary therapy for advanced (locally extensive or metastatic) prostate cancer consists of androgen deprivation therapy by pharmacotherapeutic or surgical means. Eventually, the tumor recurs owing to a transition from androgen-dependence to a highly metastatic and androgen refractory (androgen depletion-independent) phenotype. As the detailed molecular mechanism underlying this transition to a more aggressive phenotype is poorly understood, it has been difficult to develop effective treatments for this advanced stage of the disease. We have previously reported an increase in vascular endothelial growth factor-C (VEGF-C) expression in human prostate cancer cells after androgen withdrawal. We have also shown increased expression of the androgen receptor co-activator BAG-1L by VEGF-C, suggesting the involvement of this growth factor in transactivation of the androgen receptor, even at low concentrations of androgen. In our present study, we show that androgen deprivation of human prostate carcinoma cells activates the small GTPase, RalA, a molecule important for human oncogenesis. RalA activation leads to VEGF-C upregulation. We also show that elevated levels of intracellular reactive oxygen species in prostate cancer cells under androgen-ablated conditions is the major inducer of RalA activation and VEGF-C synthesis.
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Keywords: RalA; VEGF-C; androgen; reactive oxygen species; prostate cancer

Introduction

Prostate cancer is the second leading cause of cancer-related death for men in the United States. Advanced stage prostate cancer is usually treated with androgen deprivation therapy (American Cancer Society: Statistics for 2005). Although androgen deprivation therapy may modestly prolong survival, it is palliative and not curative. The vast majority of patients who initially respond to the therapy progress to the highly aggressive, androgen depletion-independent and highly metastatic stage of the disease (Kyprianou *et al*., 1990; Denis and Murphy, 1993; Epstein *et al*., 1996; Oh and Kantoff, 1998; Isaacs, 1999). It is acknowledged that vascular endothelial growth factor-C (VEGF-C) might play a role in the progression and metastatic spread of prostate tumors (Tsurusaki *et al*., 1999; Jennbacken *et al*., 2005; Zeng *et al*., 2005). VEGF-C has a role in the lymphatic vessel growth and lymph node metastasis (Karpanen *et al*., 2001; Mandriota *et al*., 2001; Skobe *et al*., 2001; He *et al*., 2002). Other studies have shown a strong correlation between VEGF-C and its receptor VEGF-R3 (flt4) expression and lymph node metastasis in human prostate carcinoma tissue (Tsurusaki *et al*., 1999; Jennbacken *et al*., 2005; Zeng *et al*., 2005). Previously, we have shown that in the prostate cancer cell line LNCaP, VEGF-C upregulation can also lead to an increase in androgen receptor co-activator, Bag-1L, suggesting that VEGF-C has the ability to transactivate androgen receptor under low androgen concentrations (Li *et al*., 2005). Taken together, these findings suggest multiple functions for VEGF-C in prostate cancer progression. Despite this evidence for the role of VEGF-C in advanced stage prostate cancer, the molecular mechanism involved in VEGF-C expression is still poorly understood. In a previous publication, we show that androgen-ablated conditions and a decrease in insulin-like growth factor 1-receptor (IGF-1R) signaling leads to activation of the forkhead transcription factor FOXO-1 and a concomitant upregulation of VEGF-C mRNA synthesis (Li *et al*., 2005). However, the IGF-1R pathway for FOXO-1 activation is not the only pathway involved in the transcriptional regulation of VEGF-C under androgen-ablated conditions. The present study demonstrates the involvement of the small GTPase RalA in the regulation of VEGF-C expression in prostate cancer.

Sequence similarity with H-, K- and N-Ras small G proteins first led to isolation of the Ras-like GTPase, RalA (Chardin and Tavitian, 1986), now considered a member of the Ras-family of GTPases. Ras family

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G-proteins are considered to be critical effectors molecules for mediating a variety of cellular responses, including proliferation, differentiation and survival. Specifically, Ral GTPases RalA and RalB have been shown to serve essential functions in vesicle trafficking, cell morphology, transcriptional activation and human oncogenesis (Feig, 2003). Studies also suggest a role for RalA in tumor metastasis (Tchevkina *et al*., 2005) and anchorage-independent proliferation (Chien and White, 2003). In contrast, activation of RalGEF and Ral Ras-effector proteins is not a potent inducer of transformation in rodent fibroblast or epithelial cells (Urano *et al*., 1996; McFall *et al*., 2001; Ulku *et al*., 2003; Collette *et al*., 2004). Taken together, these findings suggest that Ras-mediated oncogenesis occurs via distinct mechanisms in humans and mice, and highlight RalA and Ral-GEFS as unique potential targets for human cancer therapy.

A role for reactive oxygen species (ROS) in the pathogenesis of prostate cancer has also been well established. Most importantly, ROS may also induce signaling pathways involved in tumor cell survival under stress conditions. For instance, Tam *et al*. (2003) have shown that androgen deprivation leads to a significant increase in ROS production and the upregulation of ROS-generating nicotinamide adenine dinucleotide phosphate (reduced form) oxidases in rat acinar epithelial cells.

Recently, Essers *et al*. (2004) have confirmed the small GTPase RalA to be a downstream signaling molecule of ROS. They observed elevated intracellular levels of active or GTP-bound RalA in response to treatment with hydrogen peroxide, causing FOXO-4 nuclear translocation followed by transcriptional activation. These findings linking forkhead transcription factor activation with RalA activity, along with studies establishing a role for ROS and RalA in prostate tumor progression, led us to investigate a possible role for these signaling molecules in VEGF-C transcriptional regulation. Here, we describe a novel mechanism for VEGF-C transcriptional regulation in tumor-derived human prostate carcinoma cell lines.

Results

Androgen deprivation results in increased intracellular ROS generation and VEGF-C synthesis

We have previously shown that in response to androgen deprivation, VEGF-C mRNA and protein levels are elevated (~3- to 7-fold) in a time-dependent manner (Li *et al*., 2005). Following our observation that androgen negatively regulates VEGF-C synthesis, publications describing androgen deprivation-induced upregulation of intracellular ROS in the rat prostate came to our attention (Tam *et al*., 2003). Based on these published reports, we were curious to analyse whether androgen deprivation could lead to increased ROS generation in the androgen-dependent human prostate carcinoma cell line LNCaP. To do so, we cultured LNCaP cells both in normal and charcoal-dextran (charcoal stripped (CS))-treated (androgen-deprived) serum for 72 h, and then analysed ROS production by flow cytometric detection as described in Materials and methods. Under androgen-depleted conditions, we observed a significant shift in the population positive for increased ROS production, as compared to that for cells cultured in normal serum (Figure 1c). We were also able to confirm this observation by fluorescence microscopy visualization of intracellular ROS (Figure 1a and b). These data suggest that androgen deprivation leads to increased ROS generation in the LNCaP prostate carcinoma cell line.

In order to determine whether androgen deprivation-induced ROS generation is a signaling event involved in the regulation of VEGF-C mRNA level, we treated LNCaP cells cultured in CS serum with the generic ROS scavenger *N*-acetyl-cysteine (NAC). Total RNA was collected and subjected to real-time polymerase chain reaction (PCR) for quantification of VEGF-C mRNA levels. In the presence of the NAC antioxidant, we observed a reduction in VEGF-C mRNA levels, as compared to those for LNCaP cells left untreated with NAC ($P<0.05$) (Figure 2a). LNCaP cells treated with NAC were also cultured in CS serum supplemented with synthetic androgen R1881 (10 nM). As shown in Figure 2b, in the presence of synthetic androgen NAC did not show any further inhibition of VEGF-C mRNA level ($P<0.05$). These data suggest that elevation of intracellular ROS is dependent upon androgen deprivation and is a signaling event involved in the regulation of VEGF-C synthesis.

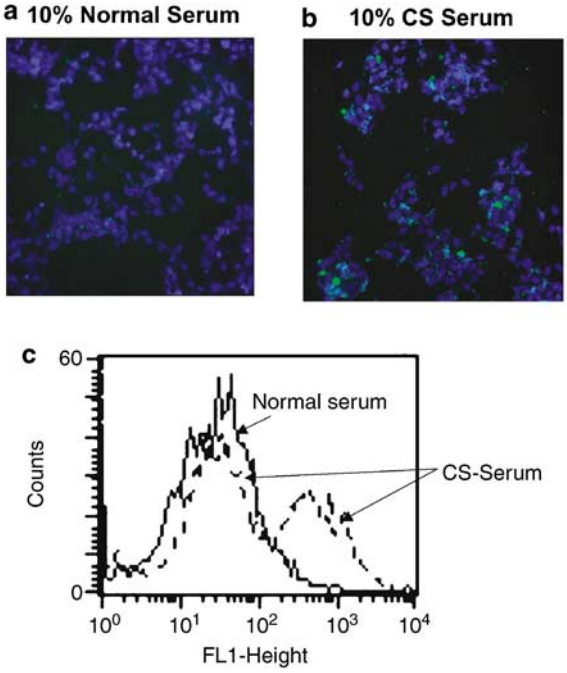

Figure 1 (**a** and **b**) LNCaP prostate carcinoma cells cultured in 10% normal and charcoal-stripped (CS, androgen-deprived) serum were subjected to ROS detection using the Image-iT LIVE Green Reactive Oxygen Species Detection Kit, followed by flow cytometric detection (**c**) or fluorescence microscopy visualization.

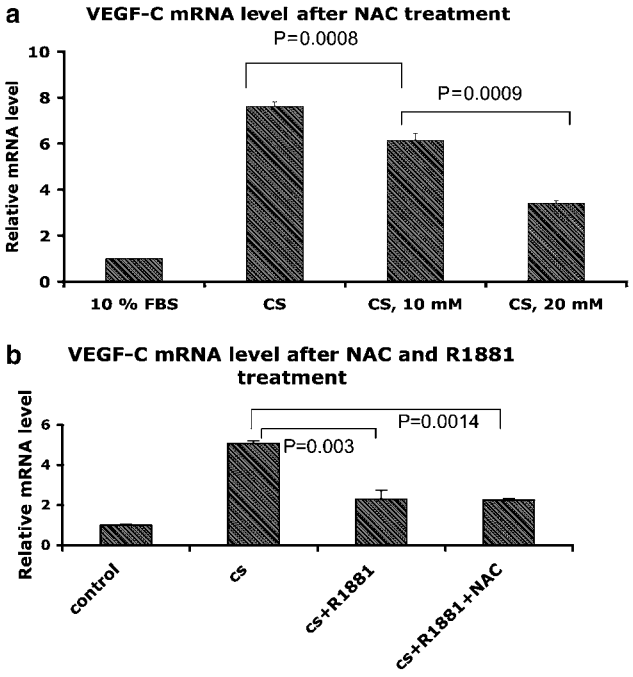

Figure 2 (**a**) LNCaP cells cultured in 10% normal and CS for 72 h were treated with 10 and 20 mM concentrations of NAC antioxidant. Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4. The data presented are the mean of three individual experiments. (**b**) LNCaP cells were cultured as above for 24 h. The cell cultures were then supplemented with R1881 synthetic androgen (10 nM) for 48 h, and then treated with the NAC antioxidant (20 mM). Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4 internal control. All data presented are the mean of three individual experiments.

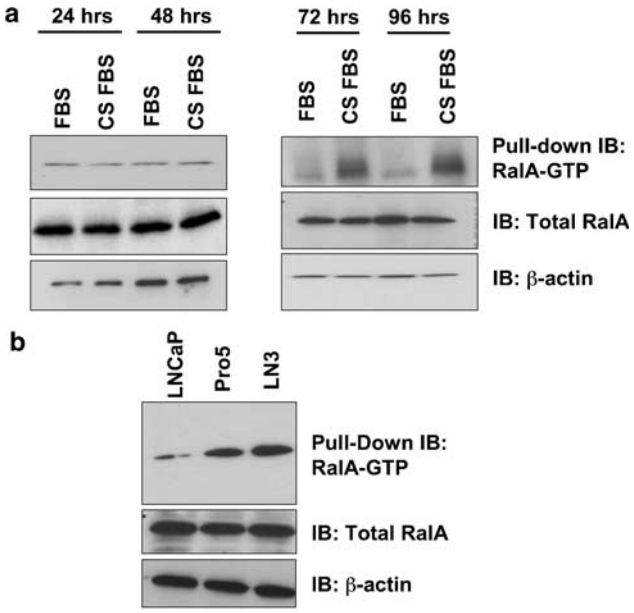

Figure 3 (**a**) LNCaP cells were cultured in 10% normal and CS for 24, 48, 72 and 96 h. Cell lysate was subjected to a pull-down assay for activated RalA-GTP using RalBP1 agarose. Western blot was performed using antibodies for RalA and β-actin internal control. (**b**) LNCaP, LNCaP-Pro5 and LNCaP-LN3 cell lines were cultured in CS for 72 h. Cell lysates were subjected to pull-down assay for activated RalA-GTP as above; Western blot carried out using antibody for RalA and β-actin internal control.

RalA is activated under androgen-ablated conditions

A report by Essers *et al*. (2004) has implicated the small GTPase RalA as a signaling molecule downstream of increased ROS generation. Recent reports implicating RalA in both human tumorigenic transformation and cancer metastasis led us to question whether RalA could be activated under conditions simulating androgen deprivation therapy, which is commonly used as treatment for advanced stage prostate cancer patients. As such, we chose to assess the involvement of RalA signaling as a downstream event for androgen deprivation-induced ROS generation. To do so, we cultured LNCaP cells under both normal and CS conditions for four different time points, and then performed a pull-down assay for activated RalA-GTP using Ral binding protein 1 (RalBP1) agarose as described in Materials and methods. Protein eluted from the RalBP1 agarose was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with antibody against RalA. We found that under androgen-deprived conditions, intracellular levels of active GTP-bound RalA were highly elevated at 72 and 96 h, as compared to RalA-GTP levels for cells under normal serum conditions (Figure 3a). Both the 24- and 48-h time points did not show significant upregulation of active RalA (Figure 3a). Total RalA levels were comparable between androgen-deprived and non-androgen-deprived cells as determined by Western blot. This finding suggests that RalA is activated within 72 h of androgen deprivation in human prostate carcinoma cells. Of note, our previous findings (Li *et al*., 2005) also support a significant upregulation of VEGF-C in LNCaP after 72 h under androgen-ablated conditions. We have also observed a detectable increase in ROS level at 72 h after androgen withdrawal (Figure 1). Therefore, taken together, these results suggest that increased synthesis of VEGF-C, ROS and activation of RalA occur at similar time points.

In response to the observation that RalA is active under androgen-ablated conditions, we chose to compare active RalA-GTP levels across several prostate carcinoma cell lines. LNCaP and LNCaP syngenic cell lines LNCaP-LN3 and LNCaP-Pro5 were chosen as representative cell lines. The syngenic Pro5 and LN3 cell lines were originally generated by harvesting LNCaP tumor cells from either the prostate or lymph nodes of athymic mice. Pro5 and LN3 characteristically exhibit higher metastatic potential than the parental cell line (LNCaP < Pro5 < LN3) (Pettaway *et al*., 1996). Interestingly, an increase in the metastatic potential of these syngenic cells lines is also associated with a decrease in their growth requirement for androgen (Pettaway *et al*., 1996). Both Pro5 and LN3 were generously provided to us by Dr Curtis A Pettaway of MD Anderson. A pull-down immunoblot assay for activated RalA-GTP was

performed with the cell lysates from LNCaP, Pro5 and LN3 as described. We observed that as compared to LNCaP, the more metastatic cell lines Pro5 and LN3 exhibited increased RalA activation (Figure 3b). In contrast, total RalA protein levels were comparable across all cell lines. This observation suggests that increasingly metastatic prostate carcinoma cell lines exhibit higher levels of RalA activation.

RalA activation is ROS signaling-dependent

Based on our previous finding that androgen deprivation increases intracellular ROS level, we chose to investigate whether RalA activation is directly regulated by ROS levels in our prostate cancer model. To do so, we cultured LNCaP cells in CS for 72 h, and then treated these cells with generic ROS scavenger NAC (20 mM). Intracellular levels of active (GTP-bound) RalA protein were measured using the pull-down immunoblot method as described previously. As expected, we observed that NAC treatment of LNCaP cells had an inhibitory effect on RalA activation (Figure 4).

VEGF-C is a downstream target of RalA signaling

Thus far, our data suggested that androgen ablation of LNCaP prostate carcinoma cells leads to increased intracellular ROS generation and subsequent RalA activation. These findings prompted us to further investigate VEGF-C as a possible downstream target for RalA signaling. Using constitutively active RalA, we first chose to study whether VEGF-C mRNA level is regulated by RalA activation. We infected LNCaP cells cultured in normal serum with a retrovirus carrying the expression vector for the constitutely active Ral Q75L (a generous gift from Dr Chenning Der). Using real-time PCR, we observed an increase in the VEGF-C mRNA level of cells infected with Ral Q75L retrovirus, as compared to those infected with the LacZ control retrovirus ($P<0.05$) (Figure 5a). As expected, infection of LNCaP cells with the Ral Q75L retrovirus led to an increase in RalA-GTP levels, as compared to the control (Figure 5b). Using the RalN28 dominant-negative expression vector kindly provided to us by Dr Johannes L Bos, we then studied whether VEGF-C mRNA level can be downregulated by inhibiting RalA activation. RalN28 is a permanently GDP-bound, dominant-negative form of RalA. LNCaP cells were cultured in CS for 24 h and then transfected for 48 h with the RalN28 dominant-negative expression vector. Total RNA was collected and subjected to real-time PCR for quantification of VEGF-C mRNA levels. Upon transfection of LNCaP cells with the RalN28 dominant-negative, we observed a statistically significant inhibition of VEGF-C mRNA level (Figure 5c). LNCaP cells transfected with the RalN28 dominant-negative expression vector showed significant expression of the vector as detected by Western blot (Figure 5d). Additionally, cells transfected with the RalN28 dominant-negative and cultured in CS supplemented with synthetic androgen R1881 did not show any further inhibition of VEGF-C mRNA level (Figure 7b). We also performed a small interfering RNA knockdown of RalA, which confirmed the inhibitory effect on VEGF-C mRNA level that we had previously observed with the RalN28 dominant-negative ($P<0.05$) (Figure 5e) (see Supplement). These findings suggest that inhibition of RalA activity has an inhibitory effect on VEGF-C mRNA level. Taken together, these findings confirm VEGF-C as a downstream target of RalA signaling, and suggest a positive correlation between RalA activation and VEGF-C synthesis.

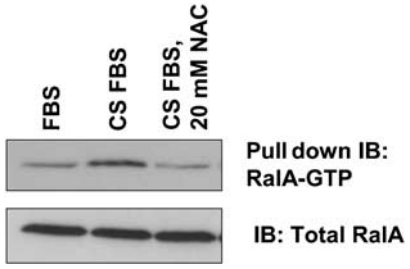

Figure 4 LNCaP cells were cultured in 10% normal and CS for 72 h. Cells cultured in CS were then treated with 20 mM NAC. Cell lysates were subjected to pull-down assay for active RalA-GTP as described. Western blot was carried out using antibody for RalA.

Androgen ablation induces sequential signaling by ROS and RalA

Based on our previous observation that treatment of LNCaP cells with NAC antioxidant could inhibit VEGF-C mRNA level under androgen-ablated conditions, we sought to confirm the possibility of a sequential ROS–RalA signaling axis. LNCaP cells cultured in CS (androgen ablated) serum were simultaneously infected with a Ral Q75L dominant-active retrovirus and treated with the NAC antioxidant as described in Materials and methods. Although treatment with NAC inhibited VEGF-C mRNA level, infection with the RalA dominant-active retrovirus restored VEGF-C mRNA levels in LNCaP cells treated with NAC ($P<0.05$) (Figure 6). Taken together, these results suggest that increased ROS generation is an upstream signaling event for RalA activation in cells under androgen-ablated conditions.

JNK activation is not required for ROS/RalA regulation of VEGF-C transcription

From previous reports, c-Jun N-terminal kinase (JNK) appears to be a likely target for ROS-induced RalA signaling (Lo *et al*., 1996; de Ruiter *et al*., 2000). As such, we chose to study whether RalA stimulation of VEGF-C synthesis is mediated by JNK. LNCaP cells were cultured for 24 h in normal and CS, and then transfected for 48 h with either a control vector or the expression vector carrying the dominant-negative form of JNK (JNK1) (kindly provided to us by Dr Roger J Davis of the University of Massachusetts). Total RNA was collected and VEGF-C mRNA levels were quantified by real-time PCR. Upon inhibition of JNK activity by the dominant-negative, we observed a decrease in

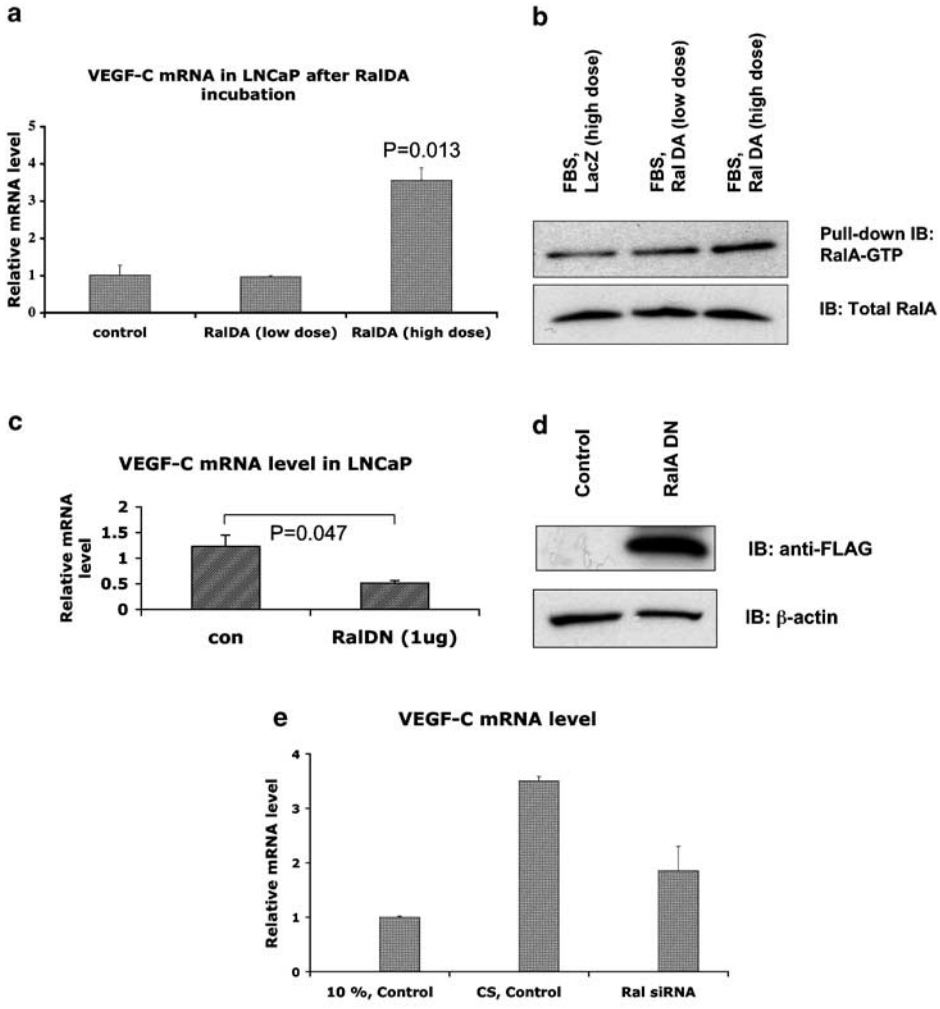

Figure 5 (**a**) LNCaP cells cultured in 10% normal serum were infected with low dose (0.4 ml) and high dose (0.8 ml) of RalA Q72L dominant-active retrovirus or LacZ control retrovirus for 48 h. Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4 internal control. The data presented here represent the mean of three individual experiments. (**b**) LNCaP cells were cultured in 10% FBS and infected with low (0.4 ml) and high (0.8 ml) dose of the RalA Q72L dominant-active retrovirus and LacZ control retrovirus for 48 h. Whole-cell lysates were collected and subjected to pull-down assay for active RalA-GTP as described. Western blot was carried out using anti-RalA antibody. (**c**) LNCaP cells cultured in 10% normal and CS conditions were transfected with 1 μg of the RalA N28 dominant-negative expression vector. After transfection for 48 h, total RNA was collected and subjected to real-time PCR using primers specific for VEGF-C and 36B4 internal control. The data presented represent the mean of three individual experiments. (**d**) LNCaP cells were transiently transfected with RalA N28 for 48 h and cell lysates were collected and resolved using SDS–PAGE. Western blots with anti-FLAG tag and β-actin antibodies were carried out as described previously. (**e**) LNCaP cells cultured in 10% normal and CS were transfected with 100 nM RalA small interfering RNA for 72 h. Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4 internal control. The data presented represent the mean of three individual experiments.

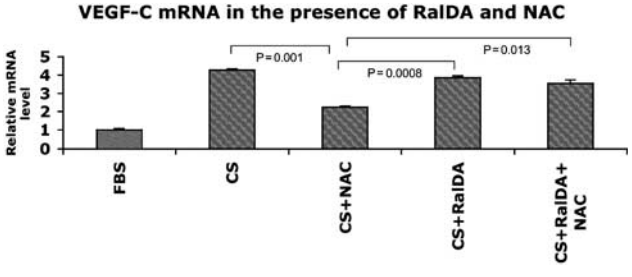

Figure 6 LNCaP cells cultured in 10% normal and CS were infected with the Ral Q72L dominant-active retrovirus for 48 h and subsequently treated with NAC antioxidant (20 mM). Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4 internal control.

VEGF-C mRNA levels for LNCaP cells cultured in androgen-deprived serum, as compared to those for LNCaP cells cultured in CS but transfected with the control vector ($P<0.05$) (Figure 7a). LNCaP cells were also transfected with the JNK1 dominant-negative and cultured in CS supplemented with R1881. As expected, addition of androgen to the cell culture did not enhance the inhibitory effect of the JNK dominant-negative on VEGF-C mRNA levels (Figure 7b). Taken together, these results suggest that JNK is involved in the upregulation of VEGF-C mRNA levels. Next, we chose to determine if JNK is in fact a downstream target of RalA signaling for VEGF-C upregulation upon

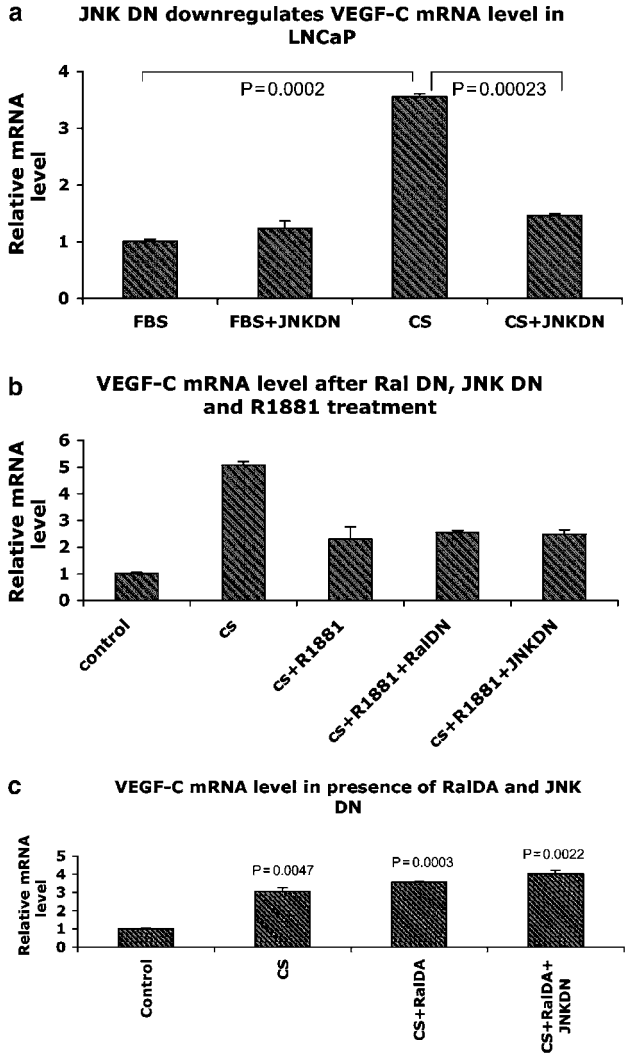

Figure 7 (**a**) LNCaP cells cultured in 10% normal and CS for 72 h were transiently transfected with the JNK-1 dominant-negative expression vector (1 μg) for 48 h. Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4 internal control. The data presented are the mean of three individual experiments. (**b**) LNCaP cells were cultured in 10% normal and CS supplemented with 10 nM R1881. The cells were also transiently transfected with the dominant-negative expression vectors for RalA and JNK for 48 h. Total RNA was collected and real-time PCR was performed using primers for VEGF-C and 36B4 internal control. The data presented are the mean of three individual experiments. (**c**) LNCaP cells were cultured as above, and simultaneously infected with RalA Q72L or LacZ control retroviruses and the JNK-1 dominant-negative expression vector for 48 h. Total RNA was collected and subjected to real-time PCR using primers for VEGF-C and 36B4. The data presented are the mean of three individual experiments.

androgen withdrawal. LNCaP cells were again cultured in both normal and CS for 24 h, infected with retroviruses carrying the Ral Q75L dominant-active and LacZ control vectors, and simultaneously transfected with the JNK-1 dominant-negative expression vector for 48 h. Real-time PCR quantification of VEGF-C mRNA levels showed that in cells infected with Ral Q75L retrovirus, transfection with the dominant-negative form of JNK did not inhibit VEGF-C mRNA levels under androgen-ablated conditions ($P<0.05$) (Figure 7c). This result suggests that JNK activation may be involved in an additional pathway converging on VEGF-C synthesis, but that it does not seem to be involved for regulation of VEGF-C mRNA levels via RalA signaling.

Discussion

Several cancers show evidence of increased VEGF-C expression during their development and progression (Kimura *et al*., 2003; Neuchrist *et al*., 2003; Jia *et al*., 2004; Sipos *et al*., 2004). Elevated VEGF-C protein levels have also been observed in human prostate cancer specimens (Tsurusaki *et al*., 1999; Jennbacken *et al*., 2005). As the molecular pathways contributing to the androgen refractory, highly metastatic stage of prostate cancer are poorly understood, the potential involvement of VEGF-C in the transition to the androgen depletion-independent phenotype of prostate cancer is an important subject area for intense research. Our previous report provided evidence for the increased expression of VEGF-C under androgen-depleted conditions, suggesting a role for this growth factor in promoting the survival of prostate tumor cells during androgen withdrawal (Li *et al*., 2005). Based on these findings, it is likely that VEGF-C expression is a critical signal involved in prostate cancer progression to the androgen depletion-independent stage. The present study sought to elucidate one of several molecular pathways leading to the high expression of VEGF-C in prostate cancer under androgen-deprived conditions.

An increase in the generation of intracellular ROS owing to androgen withdrawal has been previously reported in rat acinar epithelial cells (Tam *et al*., 2003). Here, we show that a similar increase in ROS generation also occurs in prostate cancer cells during androgen withdrawal. As a result of high intracellular levels of ROS, there may be several downstream signaling events contributing to tumorigenesis. One of them, as we observed, is RalA activation leading to VEGF-C production. Activation of RalA has been shown to be important for human cancer progression (Feig, 2003; Tchevkina *et al*., 2005). In this light, activation of RalA under androgen-ablated conditions is in itself an important observation, suggesting RalA's possible involvement in the transition of prostate cancer towards the androgen refractory phenotype.

Based on our previous observation that VEGF-C is highly expressed under androgen-ablated conditions (Li *et al*., 2005), we questioned whether RalA activation might also regulate VEGF-C expression at low levels of androgen. Our finding that RalA can regulate VEGF-C expression provides an alternative mechanism for regulation of VEGF-C that is distinctly different from that of IGF-IR signaling. To our knowledge, this is the first report suggesting that activation of RalA, an event known for its importance in human oncogenesis, is induced during androgen ablation and leads to VEGF-C

synthesis. JNK activation is a known downstream signaling event of ROS (de Ruiter *et al*., 2000). Our studies point out that JNK can regulate VEGF-C synthesis. Of note, although RalA can activate JNK in other cell types (Essers *et al*., 2004), our study suggests that in prostate cancer cells JNK is not the downstream target of RalA for VEGF-C synthesis.

Individually, ROS generation, activation of RalA and increased expression of VEGF-C have been reported by other studies to lead to cancer progression and metastasis by several known mechanisms. In the present study, we not only show that these potent tumor inducers are activated owing to androgen withdrawal in prostate cancer, but also that they are components in a unified, sequential signaling pathway for the production of an important angiogenic growth factor, VEGF-C. Taken together, our findings suggest the existence of a ROS-RalA-VEGF-C signaling axis leading to enhanced VEGF-C synthesis in prostate cancer cells – that may be important for the transition of prostate cancer from androgen dependence to the androgen refractory stage. Hypoxia is one of the strong modulators of VEGF-A synthesis in both physiological and pathological conditions. Interestingly, VEGF-C is not induced by hypoxia. Until now, very little is known about how VEGF-C expression is regulated in cancer cells. Our study is therefore significant because it elucidates a hypoxia-independent molecular pathway involved in induction of VEGF-C production, and thus paves the way for future therapeutic intervention in the treatment of advanced stage prostate cancer.

Materials and methods

Cell culture
Human prostate cancer cell line LNCaP (ATCC # CRL-1740),) and LNCaP syngenic cell lines LNCaP-Pro5 and LNCaP-LN3 were cultured at 37°C in Rosewell Park Memorial Institute medium (RPMI) 1640 with L-glutamine (Mediatech Inc., Herndon, VA, USA) supplemented with penicillin/streptomycin and containing either 10% normal fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA) or 10% charcoal-stripped (CS, androgen-depleted) FBS (Biomeda Corp., Foster City, CA, USA).

ROS detection and ROS scavenger treatment
LNCaP cells were grown in 60 mm cell culture dishes in RPMI containing either 10% normal or CS (androgen-depleted) serum for 72 h. For fluorescence microscopy visualization, cells were grown on glass coverslips placed inside the 60 mm cell culture dishes and then fixed in 4% formaldehyde phosphate-buffered saline. ROS detection was achieved using the Image-iT LIVE Green Reactive Oxygen Species Detection Kit I36007 (Invitrogen Molecular Probes, Carlesbad, CA, USA), followed by either flow cytometric detection or fluorescence microscopy visualization. For treatment with ROS scavenger, LNCaP cells were cultured in RPMI containing 10% normal or CS FBS for 72 h. The cells were then incubated with 20 mM *N*-acetyl-L-cysteine (NAC) ROS scavenger reagent (Sigma-Aldrich, Saint Louis, MO, USA) at 37.4°C for 2 h. Incubation with NAC was followed by total RNA isolation and VEGF-C mRNA quantification by real-time PCR as described below.

Active RalA detection assay
LNCaP, LNCaP-Pro5 and LNCaP-LN3 cells were grown in the presence or absence of androgen for 72 and 96 h, lysed with ice-cold radioimmunoprecipitation assay lysis buffer, incubated on ice for 10 min, and centrifuged at 10 000 r.p.m., 4°C for 10 min. The pull-down assays were performed using 0.5 mg of cellular protein from whole-cell extracts and 30 μl 50% slurry RalBP1 agarose (Upstate Biotechnology, Lake Placid, NY, USA).

Western blot analysis
The whole-cell extracts were separated by SDS–PAGE; immunodetection antibodies against RalA (Upstate Biotechnology, Lake Placid, NY, USA) and β-actin (Sigma-Aldrich) were used, followed by the secondary antibody incubation and detection by Enhanced Chemiluminescent (ESL) Substrate reagent from Amersham Biosciences Corp (Piscataway, NJ, USA).

RNA isolation and real-time PCR
RNA was isolated from LNCaP cells according to the RNeasy Minikit protocol for animal cells (Qiagen Inc., Valencia, CA, USA). The sequences for human VEGF-C and human 36B4 (housekeeping gene) were obtained from the PubMed Gene Bank and synthesized (Integrated DNA Technologies, Coralville, IA, USA) and primers were designed for real-time PCR. VEGF-C: forward, 5′-AGG GTC AGG CAG CGA ACA AGA-3′. VEGF-C: reverse, CCT CCT GAG CCA GGC ATC TG-3′. VEGF-C: middle, 5′-TGC CCC ACC AAT TAC ATG TGG AAT AAT CA-3′. 36B4: forward, 5′-ATA CAG CAG ATC CGC ATG T-3′. 36B4: reverse, 5′-TCA TGG TGT TCT TGC CCA TCA-3′. 36B4: middle, 5′-CAC CAC AGC CTT CCC GCG AA-3′. Real-time PCR was performed according to the Taqman method (see Supplement).

Statistics
For comparison between individual groups, *t*-tests with the assumptions of a two-tail distribution and two samples with equal variance were performed. A *P*-value below 0.05 was considered significant.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).